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(54) Title: NEUROPILINS AND USE THEREOF IN METHODS FOR DIAGNOSIS AND PROGNOSIS OF CANCER (57) Abstract The present invention relates to VEGF receptors (VEGFR) and neuropilins such as VEGF ₁₆₅ R/NP-1 and NP-2 that are associated with metastatic potential of a malignant cell and their use in the diagnosis and prognosis of cancer. Preferred ones are VEGF ₁₆₅ R/NP-1 and NP-2 but any neuropilin or VEGFR, where the constituents share at least about 85 % homology with either of the above VEGF ₁₆₅ R/NP-1 and NP-2 can be used. More preferably, such constituent shares at least 90 % homology. Still more preferably, each constituent shares at least 95 % homology.		

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NEUROPILINS AND USE THEREOF IN METHODS FOR DIAGNOSIS AND PROGNOSIS OF CANCER

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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5 FIELD OF THE INVENTION

The present invention provides methods for the diagnosis and prognosis of cancer, particularly metastatic cancer.

BACKGROUND OF THE INVENTION

10 Cancer, its development and treatment is a major health concern. The standard treatments available are few and directed to specific types of cancer, and provide no absolute guarantee of success. Most treatments rely on an approach that involves killing off rapidly growing cells in the hope that rapidly growing cancerous cells will succumb, either to the treatment, or at least be sufficiently reduced in numbers to
15 allow the body's system to eliminate the remainder. However most, of these treatments are non-specific to cancer cells and adversely effect non-malignant cells. Many cancers although having some phenotype relationship are quite diverse. Yet, what treatment works most effectively for one cancer may not be the best means for treating another cancer. Consequently, an appreciation of the severity of the condition
20 must be made before beginning many therapies. In order to most effective, these treatments require not only an early detection of the malignancy, but an appreciation of the severity of the malignancy. Currently, it can be difficult to distinguish cells at a molecular level as it relates to effect on treatment. Thus, methods of being able to screen malignant cells and better understand their disease state are desirable.

25 While different forms of cancer have different properties, one factor which many cancers share is that they can metastasize. Until such time as metastasis occurs,

a tumor, although it may be malignant, is confined to one area of the body. This may cause discomfort and/or pain, or even lead to more serious problems including death, but if it can be located, it may be surgically removed and, if done with adequate care, be treatable. However, once metastasis sets in, cancerous cells have invaded the body and while surgical resection may remove the parent tumor, this does not address other tumors. Only chemotherapy, or some particular form of targeting therapy, then stands any chance of success.

The process of tumor metastasis is a multistage event involving local invasion and destruction of intercellular matrix, intravasation into blood vessels, lymphatics or other channels of transport, survival in the circulation, extravasation out of the vessels in the secondary site and growth in the new location (Fidler, et al., *Adv. Cancer Res.* 28, 149-250 (1978), Liotta, et al., *Cancer Treatment Res.* 40, 223-238 (1988), Nicolson, *Biochim. Biophys. Acta* 948, 175-224 (1988) and Zetter, *N. Eng. J. Med.* 322, 605-612 (1990)). Success in establishing metastatic deposits requires tumor cells to be able to accomplish these steps sequentially. Common to many steps of the metastatic process is a requirement for motility. The enhanced movement of malignant tumor cells is a major contributor to the progression of the disease toward metastasis. Increased cell motility has been associated with enhanced metastatic potential in animal as well as human tumors (Hosaka, et al., *Gann* 69, 273-276 (1978) and Haemmerlin, et al., *Int. J. Cancer* 27, 603-610 (1981)).

Identifying factors that are associated with onset of tumor metastasis is extremely important. In addition, to using such factors for diagnosis and prognosis, those factors are an important site for identifying new compounds that can be used for treatment and as a target for treatment identifying new modes of treatment such as inhibition of metastasis is highly desirable.

Tumor angiogenesis is essential for both primary tumor expansion and metastatic tumor spread, and angiogenesis itself requires ECM degradation (Blood et al., *Biochim. Biophys. Acta* 1032:89-118 (1990)). Thus, malignancy is a systemic disease in which interactions between the neoplastic cells and their environment play a crucial role during evolution of the pathological process (Fidler, I. J., *Cancer Metastasis Rev.* 5:29-49 (1986)).

There is mounting evidence that VEGF may be a major regulator of angiogenesis (reviewed in Ferrara, et al., *Endocr. Rev.*, 13, 18-32 (1992); Klagsbrun, et al., *Curr. Biol.*, 3, 699-702 (1993); Ferrara, et al., *Biochem. Biophys. Res. Commun.*, 161, 851-858 (1989)). VEGF was initially purified from the conditioned media of folliculostellate cells (Ferrara, et al., *Biochem. Biophys. Res. Commun.*, 161, 851-858 (1989)) and from a variety of tumor cell lines (Myoken, et al., *Proc. Natl. Acad. Sci. USA*, 88:5819-5823 (1991); Plouet, et al., *EMBO. J.*, 8:3801-3806 (1991)). VEGF was found to be identical to vascular permeability factor, a regulator of blood vessel permeability that was purified from the conditioned medium of U937 cells at the same time (Keck, et al., *Science*, 246:1309-1312 (1989)). VEGF is a specific mitogen for endothelial cells (EC) *in vitro* and a potent angiogenic factor *in vivo*. The expression of VEGF is up-regulated in tissue undergoing vascularization during embryogenesis and the female reproductive cycle (Brier, et al., *Development*, 114:521-532 (1992); Shweiki, et al., *J. Clin. Invest.*, 91:2235-2243 (1993)). High levels of VEGF are expressed in various types of tumors, but not in normal tissue, in response to tumor-induced hypoxia (Shweiki, et al., *Nature* 359:843-846 (1992); Dvorak et al., *J. Exp. Med.*, 174:1275-1278 (1991); Plate, et al., *Cancer Res.*, 53:5822-5827; Ikea, et al., *J. Biol. Chem.*, 270:19761-19766 (1986)). Treatment of tumors with monoclonal antibodies directed against VEGF resulted in a dramatic reduction in tumor mass due to the suppression of tumor angiogenesis (Kim, et al., *Nature*, 382:841-844 (1993)). VEGF appears to play a principle role in many pathological states and processes related to neovascularization. Regulation of VEGF expression in affected tissues could therefore be key in treatment or prevention of VEGF induced neovascularization/angiogenesis.

VEGF exists in a number of different isoforms that are produced by alternative splicing from a single gene containing eight exons (Ferrara, et al., *Endocr. Rev.*, 13:18-32 (1992); Tischer, et al., *J. Biol. Chem.*, 806:11947-11954 (1991); Ferrara, et al., *Trends Cardio Med.*, 3:244-250 (1993); Polterak, et al., *J. Biol. Chem.*, 272:7151-7158 (1997)). Human VEGF isoforms consists of monomers of 121, 145, 165, 189, and 206 amino acids, each capable of making an active homodimer (Polterak et al., *J. Biol. Chem.*, 272:7151-7158 (1997); Houck, et al., *Mol. Endocrinol.*, 8:1806-1814

(1991)). The VEGF₁₂₁ and VEGF₁₆₅ isoforms are the most abundant. VEGF₁₂₁ is the only VEGF isoforms that does not bind to heparin and is totally secreted into the culture medium. VEGF₁₆₅ is functionally different than VEGF₁₂₁ in that it binds to heparin and cell surface heparin sulfate proteoglycans (HSPGs) and is only partially released into the culture medium (Houck, et al., *J. Biol. Chem.*, 247:28031-28037 (1992); Park, et al., *Mol. Biol. Chem.*, 4:1317-1326 (1993)). The remaining isoforms are entirely associated with cell surface and extracellular matrix HSPGs (Houck, et al., *J. Biol. Chem.*, 247:28031-28037 (1992); Park, et al., *Mol. Biol. Chem.*, 4:1317-1326 (1993)).

VEGF receptor tyrosine kinases, KDR/Flk-1 and/or Flt-1, are mostly expressed by EC (Terman, et al., *Biochem. Biophys. Res. Commun.*, 187:1579-1586 (1992); Shibuya, et al., *Oncogene*, 5:519-524 (1990); De Vries, et al., *Science*, 265:989-991 (1992); Gitay-Goran, et al., *J. Biol. Chem.*, 267:6003-6096 (1992); Jakeman, et al., *J. Clin. Invest.*, 89:244-253 (1992)). It appears that VEGF activities such as mitogenicity, chemotaxis, and induction of morphological changes are mediated by KDR/Flk-1 but not Flt-1, even though both receptors undergo phosphorylation upon binding of VEGF (Millauer, et al., *Cell*, 72:835-846 (1993); Waltenberger, et al., *J. Biol. Chem.*, 269:26988-26995 (1994); Seetharam, et al., *Oncogene*, 10:135-147 (1995); Yoshida, et al., *Growth Factors*, 7:131-138 (1996)). Recently, Soker et al., identified a new VEGF receptor which is expressed on EC and various tumor-derived cell lines such as breast cancer-derived MDA-MB-231 (231) cells (Soker, et al., *J. Biol. Chem.*, 271:5761-5767 (1996)). This receptor requires the VEGF isoform to contain the portion encoded by exon 7. For example, although both VEGF₁₂₁ and VEGF₁₆₅R bind to KDR/Flk-1 and Flt-1, only VEGF₁₆₅ binds to the new receptor.

Thus, this is an isoform-specific receptor and has been named the VEGF₁₆₅ receptor (VEGF₁₆₅R). It will also bind the 189 and 206 isoforms. VEGF₁₆₅R has a molecular mass of approximately 130 kDa, and it binds VEGF₁₆₅ with a K_d of about 2×10^{-10} M, compared with approximately 5×10^{-12} M for KDR/Flk-1. In structure-function analysis, it was shown directly that VEGF₁₆₅ binds to VEGF₁₆₅R via its exon 7-encoded domain which is absent in VEGF₁₂₁ (Soker, et al., *J. Biol. Chem.*, 271:5761-5767 (1996)). However, the function of the receptor was unclear.

Identifying the alterations in gene expression which are associated with malignant tumors, including those involved in tumor progression and angiogenesis, is clearly a prerequisite not only for a full understanding of cancer, but also to develop new rational therapies against cancer.

5 A further problem arises, in that the genes characteristic of cancerous cells are very often host genes being abnormally expressed. It is quite often the case that a particular protein marker for a given cancer while expressed in high levels in connection with that cancer is also expressed elsewhere throughout the body, albeit at reduced levels.

10 Prostatic carcinoma is the most prevalent form of cancer in males and the second leading cause of cancer death among older males (Boring, et al., *Cancer J. Clinicians*, 7-26 (1994)). Clinically, radical prostatectomy offers a patient with locally contained disease an excellent chance for cure. Unfortunately, if diagnosed after metastases are established, prostatic cancer is a fatal disease for which there is no
15 effective treatment to significantly increase survival. Recent advances in prostatic cancer diagnosis has allowed the earlier detection of human prostate cancer by use of the PSA test (Catalona, et al., *J. Urol.*, 151, 1283-1290 (1994)). Unfortunately, this early detection has not been accompanied by an improvement in determining which tumors may progress to the metastatic stage (Cookson, et al., *J. Urology* 154, 1070-
20 1073 (1995) and Aspinall, et al., *J. Urology* 154, 622-628 (1995)). Since many individuals having prostate cancer are not adversely effected by the cancer, considerable controversy has arisen as to the use of such tests. Thus, methods for early detection and early appreciation of the potential for or of the severity of the cancer, that can be taken into account in treatment of, for example, metastatic disease,
25 as well as treatment of such diseases are desirable.

SUMMARY OF THE INVENTION

We have isolated a cDNA encoding the VEGF₁₆₅ R gene (SEQ ID NO: 1) and have deduced the amino acid sequence of the receptor (SEQ ID NO:2) We have
30 discovered that this novel VEGF receptor is structurally unrelated to Flt-1 or

KDR/Flk-1 and is expressed not only by endothelial cells but by non-endothelial cells, including surprisingly tumor cells.

In ascertaining the function of the VEGF₁₆₅R we have further discovered that this receptor has been identified as a cell surface mediator of neuronal cell guidance and called neuropilin-1. Kolodkin et al., *Cell* 90:753-762 (1997). We refer to the
5 receptor as VEGF₁₆₅R/NP-1.

In addition to the expression cloning of VEGF₁₆₅R/NP-1 cDNA we isolated another human cDNA clone whose predicted amino acid sequence was 47% homologous to that of VEGF₁₆₅R/NP-1 and over 90% homologous to rat neuropilin-2
10 (NP-2) which was recently cloned (Kolodkin, et al., *Cell* 90, 753-762 (1997)). NP-2 binds members of the collapsin/semaphorin family selectively (Chen, et al., *Neuron* 19, 547-559 (1997)).

Our results indicate that VEGF₁₆₅R/NP-1 and NP-2 are expressed by both endothelial and tumor cells. (Fig. 19) We have shown that endothelial cells expressing
15 both KDR and VEGF₁₆₅R/NP-1 respond with increased chemotaxis towards VEGF₁₆₅, not VEGF₁₂₁, when compared to endothelial cells expressing KDR alone. While not wishing to be bound by theory, we believe that VEGF₁₆₅R/NP-1 functions in endothelial cells to mediate cell motility as a co-receptor for KDR.

We have also shown in the Boyden chamber motility assay that VEGF₁₆₅
20 stimulates 231 breast carcinoma cell motility in a dose-response manner (Fig 15A). VEGF₁₂₁ had no effect motility of these cells (Fig 15B). Since tumor cells such as, 231 cells, do not express the VEGF receptors, KDR or Flt-1, while not wishing to be bound by theory, we believe that tumor cells are directly responsive to VEGF₁₆₅ via VEGF₁₆₅R/NP-1.

25 We have also analyzed two variants of Dunning rat prostate carcinoma cells, AT2.1 cells, which are of low motility and low metastatic potential, and AT3.1 cells, which are highly motile, and metastatic. Cross-linking and Northern blot analysis show that AT3.1 cells express abundant VEGF₁₆₅R/NP-1, capable of binding VEGF₁₆₅, while AT2.1 cells don't express VEGF₁₆₅R/NP-1 (Fig 18). Immunostaining
30 of tumor sections confirmed the expression of VEGF₁₆₅R/NP-1 in AT3.1, but not AT2.1 tumors (Fig 17). Additionally, immunostaining showed that in subcutaneous

AT3.1 and PC3 tumors, the tumor cells expressing VEGF₁₆₅R/NP-1 were found preferentially at the invading front of the tumor/dermis boundary (Fig 17).

Furthermore, stable clones of AT2.1 cells overexpressing VEGF₁₆₅R/NP-1 had enhanced motility in the Boyden chamber assay. These results indicate that

5 VEGF₁₆₅R/NP-1 expression on tumor cells is associated with the motile, metastatic phenotype.

These results indicate that enhanced transcripts (mRNA) and expression of the VEGF₁₆₅R/NP-1 and NP-2 receptors have a high correlation to disease state in a number of cancers, such as prostate, hemangioendothelioma and breast, particularly
10 metastatic cancers. Accordingly, assaying for enhanced levels of transcript or gene product can be used in not only a diagnostic manner, but in a prognostic manner for particular cancers. Additionally, by blocking such receptors or inhibiting their occurrence, one can inhibit metastasis.

The present invention provides a method of diagnosing cancer, especially
15 prostate cancer, breast cancer, and hemangioendothelioma in a patient by measuring levels of VEGF₁₆₅R/NP-1 or NP-2 in a biological specimen obtained from the patient. Levels of VEGF₁₆₅R/NP-1 or NP-2 in the sample greater than a base line level for that type of specimen is indicative of cancer. Biological specimens include, for example, blood, tissue, serum, stool, urine, sputum, cerebrospinal fluid and supernatant from
20 cell lysate. The determination of base lines and comparison levels is by standard modes of analysis based upon the present disclosure.

In another aspect, the present invention provides a method of prognosis in an individual having cancer, the method comprising:

- a. obtaining a tumor sample from said individual;
- 25 b. measuring VEGF₁₆₅R/NP-1 or NP-2 amounts to obtain an VEGF₁₆₅R/NP-1 level in said sample;
- c. correlating said VEGF₁₆₅R/NP-1 levels with a baseline level; and correlating levels of VEGF₁₆₅R/NP-1 or NP-2 higher than the baseline with an indication of unfavorable prognosis and levels of VEGF₁₆₅R/NP-1 or NP-2 at the
30 baseline or less with a favorable prognosis. VEGF₁₆₅R/NP-1 mRNA or protein may be measured to obtain VEGF₁₆₅R/NP-1 levels.

In accordance with the present invention, expression of VEGF₁₆₅R/NP-1 or NP-2 in a tumor sample greater than a base line level for that particular tissue indicates a higher risk of tumor metastasis.

5 In yet another aspect, the present invention provides a method for determining the metastatic potential of a tumor by measuring the level of VEGF₁₆₅R/NP-1 or NP-2 expression in the tumor. Expression of VEGF₁₆₅R/NP-1 or NP-2 in said tumor greater than a base line level for that particular tissue indicates an increased metastatic potential.

10 In yet another embodiment, changes in condition can be monitored by comparing changes in VEGF₁₆₅R/NP-1 or NP-2 expression levels in the tumor in that subject over time.

In the methods of the present invention, levels of VEGF₁₆₅R/NP-1 or NP-2 can be ascertained by measuring the protein directly or indirectly by measuring transcript (mRNA) encoding VEGF₁₆₅R/NP-1 or NP-2. mRNA levels can be measured, for
15 example, using an RNA dependent polymerase chain reaction, e.g., reverse transcriptase PCR or Northern blot analysis. DNA chip technology may also be used to measure mRNA levels.

Base line levels can readily be determined by measuring levels of VEGF₁₆₅R/NP-1 or NP-2 in sample of disease free individuals.

20 The present invention also provides of a method for measuring VEGF₁₆₅R/NP-1 or NP-2 levels in non-neuronal tissues which comprises the steps of:

- a. contacting a biological specimen with an antibody or antibody fragment which selectively binds VEGF₁₆₅R/NP-1 or NP-2, and
- b. detecting whether said antibody or said antibody fragment is bound by said
25 sample and thereby measuring the levels of VEGF₁₆₅R/NP-1 or NP-2.

In still another embodiment of this invention, the receptor can serve as a target for compounds that disrupt its function. Such compounds include, for example, VEGF antagonists, compounds that bind to NP-1 or NP-2 and antibodies that specifically binds the receptor at a region that inhibits receptor function. For example, one can add
30 an effective amount of a compound that binds to NP-1 to disrupt receptor and thus inhibit metastasis. In another embodiment, one can use such VEGF₁₆₅R/NP-1 or NP-2

cells in an assay to discover compounds that bind to or otherwise interact with these receptors in order to discover compounds that can be used to inhibit metastasis.

Other aspects of the invention are disclosed *infra*.

5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows purification of VEGF₁₆₅R From 231 Cells.

¹²⁵I-VEGF₁₆₅ (5 ng/ml) was bound and cross-linked to receptors on 231 cells and analyzed by SDS PAGE and autoradiography (lane 1). VEGF₁₆₅R was purified by Con A and VEGF₁₆₅ affinity column chromatography and analyzed by SDS-PAGE
10 and silver stain (lane 2). Two prominent bands were detected (arrows) and N-terminally sequenced separately. Their N-terminal 18 amino acid sequences are shown to the right of the arrows. The published N-terminal sequences of human and mouse neuropilin (Kawakami et al., *J. Neurobiol.*, 29, 1-17 (1995); He and Tessier-Lavigne, *Cell* 90, 739-751 1997) are shown below (SEQ ID NOS: 5 and 6).

15

Figures 2A and 2B show isolation of VEGF₁₆₅R cDNA by Expression Cloning. Photomicrographs (dark field illumination) of COS 7 cells binding ¹²⁵I-VEGF₁₆₅. ¹²⁵I-VEGF₁₆₅ was bound to transfected COS 7 cells which were then washed, fixed, and overlaid with photographic emulsion that was developed as described in the
20 example, *infra*.

Figure 2A shows COS 7 cells were transfected with a primary plasmid pool (#55 of the 231 cell library) representing approximately 3 x 10³ clones and one COS 7 cell binding ¹²⁵I-VEGF₁₆₅ in the first round of screening is shown.

Figure 2 shows several COS 7 cells transfected with a single positive cDNA
25 clone (A2) binding ¹²⁵I-VEGF₁₆₅ after the third round of screening.

Figure 3 shows the Deduced Amino Acid Sequence of Human VEGF₁₆₅R/NP-1 (SEQ ID NO:3). The deduced 923 amino acid sequence of the open reading frame of VEGF₁₆₅R/NP-1, clone A2 (full insert size of 6.5 kb) is shown. The putative signal peptide sequence (amino acids 1-21) and the putative transmembrane region (amino
30 acids 860-883) are in boxes. The amino acid sequence obtained by N-terminal amino

acid sequencing (Figure 3, amino acids 22-39) is underlined. The arrow indicates where the signal peptide has been cleaved and removed, based on comparison of the N-terminal sequence of purified VEGF₁₆₅R/NP-1 and the cDNA sequence. The sequence of human VEGF₁₆₅R/NP-1 reported here differs from that reported by He et al. (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997)) in that we find Lys₂₆ rather than Glu₂₆, and Asp₈₅₅ rather than Glu₈₅₅. Lys₂₆ and Asp₈₅₅ are found, however, in mouse and rat VEGF₁₆₅R/NP-1 (Kwakami et al., *J. Neurobiol.* 29, 1-17 (1995); He and Tessier-Lavigne, *Cell* 90, 739-751 1997).

10 Figure 4 shows the Comparison of the Deduced Amino Acid Sequence of Human VEGF₁₆₅R/NP-1 (SEQ ID NO:2) and NP-2 (SEQ ID NO:4). The deduced open reading frame amino acid sequences of VEGF₁₆₅R/NP-1 and NP-2 are aligned using the DNASIS program. Amino acids that are identical in both open reading frames are shaded. The overall homology between the two sequences is 43%.

15 Figure 5 shows a Northern Blot Analysis of VEGF₁₆₅R/NP-1 Expression in Human EC and Tumor-Derived Cell Lines. Total RNA samples prepared from HUVEC (lane 1) and tumor-derived breast carcinoma, prostate carcinoma and melanoma cell lines as indicated (lanes 2-8) were resolved on a 1% agarose gel and
20 blotted onto a GeneScreen nylon membrane. The membrane was probed with ³²P-labeled VEGF₁₆₅R/NP-1 cDNA and exposed to X-ray film. Equal RNA loading was demonstrated by ethidium bromide staining of the gel prior to blotting. A major species of VEGF₁₆₅R/NP-1 mRNA of approximately 7 kb was detected in several of the cell lines.

25 Figure 6 shows a Northern Blot Analysis of VEGF₁₆₅R/NP-1 and KDR mRNA in Adult Human Tissues. A pre-made Northern blot membrane containing multiple samples of human mRNA (Clontech) was probed with ³²P-labeled VEGF₁₆₅R/NP-1 cDNA (top) as described in Fig 5, and then stripped and reprobed with ³²P-labeled
30 KDR cDNA (bottom).

Figures 7A and 7B show a Scatchard Analysis of VEGF₁₆₅ binding to VEGF₁₆₅R/NP-1. Figure 7A. Increasing amounts of ¹²⁵I-VEGF₁₆₅ (0.1-50 ng/ml) were added to subconfluent cultures of PAE cells transfected with human VEGF₁₆₅R/NP-1 cDNA (PAE/NP-1 cells) in 48 well dishes. Non-specific binding was determined by competition with a 200-fold excess of unlabeled VEGF₁₆₅. After binding, the cells were washed, lysed and the cell-associated radioactivity was determined using a γ counter.

Figure 7B. The binding data shown in Figure 7A were analyzed by the method of Scatchard, and a best fit plot was obtained with the LIGAND program (Munson and Rodbard, 1980). PAE/NP-1 cells express approximately 3 X 10⁵ VEGF₁₆₅ binding sites per cell and bind ¹²⁵I-VEGF₁₆₅ with a K_d of 3.2 X 10⁻¹⁰ M.

Figure 8 shows cross-linking of VEGF₁₆₅ and VEGF₁₂₁ to PAE cells Expressing VEGF₁₆₅R/NP-1 and/or KDR. ¹²⁵I-VEGF₁₆₅ (5 ng/ml) (lanes 1-6) or ¹²⁵I-VEGF₁₂₁ (10 ng/ml) (lanes 7-10) were bound to subconfluent cultures of HUVEC (lane 1), PC3 (lane 2), PAE (lanes 3 and 7), a clone of PAE cells transfected with human VEGF₁₆₅R/NP-1 cDNA (PAE/NP-1) (lanes 4 and 8), a clone of PAE cells transfected with KDR (PAE/KDR) (lanes 5 and 9), and a clone of PAE/KDR cells transfected with human VEGF₁₆₅R/NP-1 cDNA (PAE/KDR/NP-1) (lanes 6 and 10). The binding was carried out in the presence of 1 μg/ml heparin. At the end of a 2 hour incubation, each ¹²⁵I-VEGF isoform was chemically cross-linked to the cell surface. The cells were lysed and proteins were resolved by 6% SDS-PAGE. The polyacrylamide gel was dried and exposed to X-ray film. Solid arrows denote radiolabeled complexes containing ¹²⁵I-VEGF and KDR, open arrows denote radiolabeled complexes containing ¹²⁵I-VEGF and VEGF₁₆₅R/NP-1.

Figure 9 shows cross linking of VEGF₁₆₅ to PAE/KDR Cells Co-expressing VEGF₁₆₅R/NP-1 Transiently. PAE/KDR cells were transfected with pCPhygro or pCPhyg-NP-1 plasmids as described in "Experimental Procedures", and grown for 3 days in 6 cm dishes. ¹²⁵I-VEGF₁₆₅ (5 ng/ml) was bound and cross linked to parental

PAE/KDR cells (lane 1), to PAE/KDR cells transfected with pCPhygro vector control (V) (lane 2), to PAE/KDR cells transfected with pCPhygro- VEGF₁₆₅R/NP-1 plasmids (VEGF₁₆₅R/NP-1) (lane 3), and to HUVEC (lane 4).). The binding was carried out in the presence of 1 µg/ml heparin. The cells were lysed and proteins were resolved by 6% SDS-PAGE as in Figure 8. Solid arrows denote radiolabeled complexes containing ¹²⁵I-VEGF₁₆₅ and KDR. Open arrows denote radiolabeled complexes containing ¹²⁵I-VEGF₁₆₅ and VEGF₁₆₅R/NP-1.

Figure 10 shows inhibition of ¹²⁵I-VEGF₁₆₅ binding to VEGF₁₆₅R/NP-1 interferes with its binding to KDR. ¹²⁵I-VEGF₁₆₅ (5 ng/ml) was bound to subconfluent cultures of PAE transfected with human VEGF₁₆₅R/NP-1 cDNA (PAE/NP-1) (lanes 1 and 2), PAE/KDR cells (lanes 3 and 4), and PAE/KDR cells transfected with human VEGF₁₆₅R/NP-1 cDNA (PAE/KDR/NP-1) (lanes 5 and 6) in 35 mm dishes. The binding was carried out in the presence (lanes 2, 4, and 6) or the absence (lanes 1, 3, and 5) of 25 µg/ml GST-Ex 7+8. Heparin (1 µg/ml) was added to each dish. At the end of a 2 hour incubation, ¹²⁵I-VEGF₁₆₅ was chemically cross-linked to the cell surface. The cells were lysed and proteins were resolved by 6% SDS-PAGE as in Figure 9. Solid arrows denote radiolabeled complexes containing ¹²⁵I-VEGF₁₆₅ and KDR, open arrows denote radiolabeled complexes containing ¹²⁵I-VEGF₁₆₅ and VEGF₁₆₅R/NP-1.

Figures 11A-C show a model for VEGF₁₆₅R/NP-1 modulation of VEGF₁₆₅ Binding to KDR. 11A.Cells expressing KDR alone. 11B.Cells co-expressing KDR and VEGF₁₆₅R/NP-1. 11C.Cells co-expressing KDR and VEGF₁₆₅R/NP-1 in the presence of GST- Ex 7+8 fusion protein.

A single KDR receptor or a KDR-VEGF₁₆₅R/NP-1 pair is shown in top portion. An expanded view showing several receptors is shown in the bottom portion. VEGF₁₆₅ binds to KDR via exon 4 and to VEGF₁₆₅R/NP-1 via exon 7 (Keyt et al. *J. Biol. Chem.* 271,5638-5646 (1996b); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). A rectangular VEGF₁₆₅ molecule represents a suboptimal conformation that doesn't bind to KDR efficiently while a rounded VEGF₁₆₅ molecule represents one

that fits better into a binding site. In cells expressing KDR alone, VEGF₁₆₅ binds to KDR in a sub-optimal manner. In cells co-expressing KDR and VEGF₁₆₅R/NP-1, the binding efficiency of VEGF₁₆₅ to KDR is enhanced. It may be that the presence of VEGF₁₆₅R/NP-1 increases the concentration of VEGF₁₆₅ on the cell surface, thereby
5 presenting more growth factor to KDR. Alternatively, VEGF₁₆₅R/NP-1 may induce a change in VEGF₁₆₅ conformation that allows better binding to KDR, or both might occur. In the presence of GST-Ex 7+8, VEGF₁₆₅ binding to VEGF₁₆₅R/NP-1 is competitively inhibited and its binding to KDR reverts to a sub-optimal manner.

10 Figure 12 shows the human NP-2 amino acid sequence (SEQ ID NO:4).

Figures 13A and 13B show the human NP-2 DNA sequence (SEQ ID NO:3).

Figures 14A, 14B and 14C show the nucleotide (SEQ ID NO:1) and amino
15 acid sequences (SEQ ID NO:2) of VEGF₁₆₅R/NP-1.

Figures 15A and 15B show VEGF₁₆₅ stimulation of MDA MB 231 cell motility. (Figure 15A) Dose response of VEGF₁₆₅ motility activity. (Figure 15B) Both VEGF₁₆₅ and bFGF stimulate motility but VEGF₁₂₁ does not.

20

Figures 16A, 16B and 16C show motility and neuropilin-1 expression of Dunning rat prostate carcinoma cell lines AT3-1 (high motility, high metastatic potential) and AT2.1 (low motility, low metastatic potential) cells. (Figure 16A) AT3.1 cells are more motile than AT2.1 cells in a Boyden chamber assay. (Figure
25 16B) ¹²⁵I-VEGF₁₆₅ cross-links neuropilin-1 on AT3.1 cells but does not cross-link to AT2.1 cells. (Figure 16C) AT3.1 but not AT2.1 cells express neuropilin-1, while both cell types express VEGF.

Figures 17A, 17B and 17C show immunostaining of (Figure 17A) a PC3
30 subcutaneous tumor in a nude mouse, (Figure 17B) an AT3.1 tumor in a rat, (Figure 17C) an AT2.1 tumor in rat with anti-neuropilin-1 antibodies. Neuropilin

immunostaining is preferentially associated with PC3 and AT3.1 tumor cells at the tumor/dermis boundary. Some of these cells cluster around blood vessels. AT2.1 cells do not express neuropilin-1.

5 Figures 18A and 18B show overexpression of neuropilin-1 in AT2.1 cells. (Figure 18A) Western blot, (Figure 18B) motility activity. Three AT2.1 clones (lanes 4,5,6) express higher amounts of neuropilin-1 protein and are more motile compared to parental AT2.1 cells or AT2.1 vector (AT2.1/V) controls and approach AT3.1 cell neuropilin-1 levels and migration activity.

10

Figure 19 shows expression of NP-1, NP-2 and β -actin in cancer cell lines and endothelial cells using reverse transcriptase PCR following primers:

Human NP-1

Forward (328-351): 5' TTTCGCAACGATAAATGTGGCGAT 3' (SEQ ID NO:7)

15 Reverse (738-719): 5' TATCACTCCACTAGGTGTTG 3' (SEQ ID NO:8)

Human NP-2

Forward (513-532): 5' CCAACCAGAAGATTGTCCTC 3' (SEQ ID NO:9)

Reverse (1181-1162): 5' GTAGGTAGATGAGGCACTGA 3'. (SEQ ID NO:10)

20

DETAILED DESCRIPTION OF THE INVENTION

We have discovered that there are VEGF receptors (VEGFR) and neuropilins such as VEGF₁₆₅R/NP-1 and NP-2 that are associated with metastatic potential of a malignant cell. Preferred ones are VEGF₁₆₅R/NP-1 and NP-2 but any neuropilin or VEGFR, where the constituents share at least about 85% homology with either of the above VEGF₁₆₅R/NP-1 and NP-2 can be used. More preferably, such constituent shares at least 90% homology. Still more preferably, each constituent shares at least 95% homology.

Homology is measured by means well known in the art. For example % homology can be determined by any standard algorithm used to compare homologies. These include, but are not limited to BLAST 2.0 such as BLAST 2.0.4 and i. 2.0.5 available from the NIH (See www.ncbi.nlm.nih.gov/BLAST/newblast.html) (Altschul, S.F., et al. Nucleic Acids Res. 25: 3389-3402 (1997)) and DNASIS (Hitachi

30

Software Engineering America, Ltd.). These programs should preferably be set to an automatic setting such as the standard default setting for homology comparisons. As explained by the NIH, the scoring of gapped results tends to be more biologically meaningful than ungapped results.

5 For ease of reference, this disclosure will generally talk about VEGF₁₆₅R/NP-1 and NP-2 and/or homologs thereof but all teaching are applicable to the above-described homologs.

In another embodiment a VEGFR can be used as long as it binds to a sequence having at least 90%, more preferably 95% homology to exon 7 of VEGF₁₆₅.

10 These VEGF receptors and neuropilins, e.g., VEGF₁₆₅R/NP-1 and NP-2, are associated with both tumor metastases and angiogenesis. We have shown that expression of VEGF₁₆₅R/NP-1 and NP-2 is upregulated in highly metastatic prostate cancer cell lines relative to poorly metastatic or nonmetastatic lines. Thus, expression of VEGF₁₆₅R/NP-1 and NP-2 can be used to determine metastatic potential. In
15 addition, the receptor and the component provide a target for treatments to inhibit the metastasis process.

Accordingly, the evaluation and comparison of levels of transcript (mRNA) or gene product, either normal or mutated, in non-neuronal tissue can be both diagnostic and prognostic of particular cancer. For example, an elevated level is indicative of a
20 greater tendency for metastatic activity. Conversely, lower levels than certain baselines can also be used to indicate the metastatic potential of the tumor. Further, by monitoring a particular neoplastic growth over a period of time and comparing changes in level one can evaluate changes in metastatic activity.

The present invention provides a method of diagnosing cancer, preferably
25 prostate and breast cancer, in a patient by measuring levels of VEGF₁₆₅R/NP-1 or NP-2 in a biological specimen obtained from the patient. Levels of VEGF₁₆₅R/NP-1 or NP-2 in the sample greater than a base line level is indicative of cancer. Baseline levels can readily be determined by measuring levels of VEGF₁₆₅R/NP-1 or NP-2 in a sample of disease free individuals. Additionally, baselines can be obtained by
30 measuring individuals having cancer over the course of the malignancy.

Biological specimens include, for example, blood, tissue, serum, stool, urine, sputum, cerebrospinal fluid and supernatant from cell lysate. Preferably, one uses tissue specimens. The determination of base lines and comparison levels is by standard modes of analysis based upon the present disclosure.

5 The present invention also provides a method of prognosis in an individual having cancer, preferable prostate, breast cancer and melanoma by measuring levels of VEGF₁₆₅R/NP-1 or NP-2 in a tumor sample obtained from a patient to be tested. Expression of VEGF₁₆₅R/NP-1 or NP-2 in said tumor sample greater than a base line level for that particular tissue indicates a higher risk of tumor metastasis. This
10 information can be used by the physician in determining the most effective course of treatment.

Changes in a patients condition can be monitored using the methods of the present invention by comparing changes in VEGF₁₆₅R/NP-1 or NP-2 expression levels in the tumor in that subject over time. For example, determining whether the level
15 stabilizes or preferably declines.

The present invention further provides a method for determining the metastatic potential of a tumor by measuring the level of VEGF₁₆₅R/NP-1 or NP-2 expression in the tumor. Expression of VEGF₁₆₅R/NP-1 or NP-2 in said tumor greater than a base line level for that particular tissue indicates an increased metastatic potential.

20 Standard detection techniques well known in the art for detecting RNA, DNA, proteins and peptides can readily be applied to detect VEGF₁₆₅R/NP-1 or NP-2 or its transcript to diagnose cancer, especially metastatic cancer or to confirm that a primary tumor has, or has not, reached a particular metastatic phase.

Such techniques may include detection with nucleotide probes or may
25 comprise detection of the protein by, for example, antibodies or their equivalent. Preferably, the nucleotide probes hybridize to the sequence shown in SEQ ID NO:1 for VEGF₁₆₅R/NP-1 and SEQ ID NO:3 for NP-2. A VEGFR/NP-1 homolog has at least about 85% homology to the amino acid sequence encoded by SEQ ID NO:1, whereas a VEGFR/NP-2 homolog has at least about 85% homology to the amino acid
30 sequence encoded by SEQ ID NO:3.

Types of probe include cDNA, riboprobes, synthetic oligonucleotides and genomic probes. The type of probe used will generally be dictated by the particular situation, such as riboprobes for in situ hybridization, and cDNA for Northern blotting, for example. The most preferred probes are those which correspond to the cytoplasmic domain of the receptor. Most preferably, the probe is directed to nucleotide regions
5 unique to the receptor. Detection of the VEGF₁₆₅R/NP-1 or NP-2 encoding gene, per se, will be useful in screening for mutations associated with enhanced expression. Other forms of assays to detect targets more readily associated with levels of expression--transcripts and other expression products will generally be useful as well.
10 The probes may be as short as is required to differentially recognize VEGF₁₆₅R/NP-1 or NP-2 mRNA transcripts, and may be as short as, for example, 15 bases, however, probes of at least 17 bases, more preferably 18 bases and still more preferably 20 bases are preferred.

A probe may also be reverse-engineered by one skilled in the art from the
15 amino acid sequence of SEQ ID NO:2 (VEGF₁₆₅R/NP-1) or SEQ ID NO:4 (NP-2). However use of such probes may be more limited than the native DNA sequence, as it will be appreciated that any one given reverse-engineered sequence will not necessarily hybridize well, or at all with any given complementary sequence reverse-engineered from the same peptide, owing to the degeneracy of the genetic
20 code. This is a factor common in the calculations of those skilled in the art, and the degeneracy of any given sequence is frequently so broad as to yield a large number of probes for any one sequence.

The form of labeling of the probes may be any that is appropriate, such as the use of radioisotopes, for example, ³²P and ³⁵S. Labeling with radioisotopes may be
25 achieved, whether the probe is synthesized chemically or biologically, by the use of suitably labeled bases. Other forms of labeling may include enzyme or antibody labeling such as is characteristic of ELISA.

Detection of RNA transcripts may be achieved by Northern blotting, for example, wherein a preparation of RNA is run on a denaturing agarose gel, and
30 transferred to a suitable support, such as activated cellulose, nitrocellulose or glass or

nylon membranes. radiolabeled cDNA or RNA is then hybridized to the preparation, washed and analyzed by autoradiography.

In situ hybridization visualization may also be employed, wherein a radioactively labeled antisense cRNA probe is hybridized with a thin section of a biopsy sample, washed, cleaved with RNase and exposed to a sensitive emulsion for autoradiography. The samples may be stained with haematoxylon to demonstrate the histological composition of the sample, and dark field imaging with a suitable light filter shows up the developed emulsion. Non-radioactive labels such as digoxigenin may also be used.

Immunohistochemistry may be used to detect expression of human VEGF₁₆₅R/NP-1 or NP-2 in a biopsy sample. A suitable antibody is brought into contact with, for example, a thin layer of cells, washed, and then contacted with a second, labeled antibody. Labeling may be by enzyme, such as peroxidase, avidin or by radiolabelling. Chromogenic labels are generally preferable, as they can be detected under a microscope.

More generally preferred is to detect the protein by immunoassay, for example by ELISA or RIA, which can be extremely rapid. Thus, it is generally preferred to use antibodies, or antibody equivalents, to detect VEGF₁₆₅R/NP-1 or NP-2.

It may not be necessary to label the substrate, provided that the product of the enzymatic process is detectable and characteristic in its own right (such as hydrogen peroxide for example). However, if it is necessary to label the substrate, then this may also comprise enzyme labeling, labeling with radioisotopes, antibody labeling, fluorescent marker labeling or any other suitable form which will be readily apparent to those skilled in the art.

Antibodies may be prepared as described below, and used in any suitable manner to detect expression of VEGF₁₆₅R/NP-1 or NP-2. Antibody-based techniques include ELISA (enzyme linked immunosorbent assay) and RIA (radioimmunoassay). Any conventional procedures may be employed for such immunoassays. The procedures may suitably be conducted such that: a VEGF₁₆₅R/NP-1 or NP-2 standard is labeled with a radioisotope such as ¹²⁵I or ³⁵S, or an assayable enzyme, such as horseradish peroxidase or alkaline phosphatase and, together with the unlabelled

sample, is brought into contact with the corresponding antibody, whereon a second antibody is used to bind the first and radioactivity or the immobilized enzyme assayed (competitive assay); alternatively, VEGF₁₆₅R/NP-1 or NP-2 in the sample is allowed to react with the corresponding immobilized antibody, radioisotope- or
5 enzyme-labeled anti-receptor antibody is allowed to react with the system and radioactivity or the enzyme assayed (ELISA-sandwich assay). Other conventional methods may also be employed as suitable.

The above techniques may be conducted essentially as a "one-step" or "two-step" assay. The "one-step" assay involves contacting antigen with immobilized
10 antibody and, without washing, contacting the mixture with labeled antibody. The "two-step" assay involves washing before contacting the mixture with labeled antibody. Other conventional methods may also be employed as suitable.

Enzymatic and radio-labeling of receptor and/or the antibodies may be effected by conventional means. Such means will generally include covalent linking of the
15 enzyme to the antigen or the antibody in question, such as by glutaraldehyde, specifically so as not to adversely affect the activity of the enzyme, by which is meant that the enzyme must still be capable of interacting with its substrate, although it is not necessary for all of the enzyme to be active, provided that enough remains active to permit the assay to be effected. Indeed, some techniques for binding enzyme are
20 non-specific (such as using formaldehyde), and will only yield a proportion of active enzyme.

It is usually desirable to immobilize one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with the component and readily removed without laborious and time-consuming labor.
25 It is possible for a second phase to be immobilized away from the first, but one phase is usually sufficient.

It is possible to immobilize the enzyme itself on a support, but if solid-phase enzyme is required, then this is generally best achieved by binding to antibody and affixing the antibody to a support, models and systems for which are well-known in
30 the art. Simple polyethylene may provide a suitable support.

Enzymes employable for labeling are not particularly limited, but may be selected from the members of the oxidase group, for example. These catalyze production of hydrogen peroxide by reaction with their substrates, and glucose oxidase is often used for its good stability, ease of availability and cheapness, as well as the ready availability of its substrate (glucose). Activity of the oxidase may be assayed by measuring the concentration of hydrogen peroxide formed after reaction of the enzyme-labeled antibody with the substrate under controlled conditions well-known in the art.

Other techniques may be used to detect VEGF₁₆₅R/NP-1 or NP-2 according to a practitioner's preference based upon the present disclosure. One such technique is Western blotting (Towbin et al., *Proc. Nat. Acad. Sci.* 76:4350 (1979)), wherein a suitably treated sample is run on an SDS-PAGE gel before being transferred to a solid support, such as a nitrocellulose filter. Anti-receptor antibodies (unlabelled) are then brought into contact with the support and assayed by a secondary immunological reagent, such as labeled protein A or anti-immunoglobulin (suitable labels including ¹²⁵I, horseradish peroxidase and alkaline phosphatase).

Samples for diagnostic purposes may be obtained from any number of sources. A sample obtained direct from the tumor, such as the stroma or cytosol, may be used to determine the metastatic potential of the tumor. It may also be appropriate to obtain the sample from other biological specimens, such as blood or urine. Such diagnosis may be of particular importance in monitoring progress of a patient, such as after surgery to remove a tumor. If a reference reading is taken after the operation, then another taken at regular intervals, any rise could be indicative of a relapse, or possibly a metastasis. Preferably, the sample is from the tumor itself.

The antibodies may be raised against either a peptide of the receptor or the whole molecule. Such a peptide may be presented together with a carrier protein, such as an KLH, to an animal system or, if it is long enough, say 25 amino acid residues, without a carrier.

Polyclonal antibodies generated by the above technique may be used direct, or suitable antibody producing cells may be isolated from the animal and used to form a

hybridoma by known means (Kohler and Milstein, *Nature* 256:795. (1975)). Selection of an appropriate hybridoma will also be apparent to those skilled in the art, and the resulting antibody may be used in a suitable assay to identify VEGF₁₆₅R/NP-1 or NP-2.

5 This invention provides a convenient kit for measuring human VEGF₁₆₅R/NP-1 or NP-2 and their homologs. This kit includes antibodies or antibody fragments which selectively bind human VEGF₁₆₅R/NP-1 or a set of DNA oligonucleotide primers that allow synthesis of cDNA encoding the receptors. Preferably, the primers comprise at least 17 nucleotides and hybridizes under stringent conditions to a DNA
10 fragment having the nucleotide sequence set forth in SEQ ID NO:1 (VEGF₁₆₅R/NP-1) or SEQ ID NO:3 (NP-2). As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences.

 DNA encoding human VEGF₁₆₅R/NP-1 or NP-2 and recombinant human
15 VEGF₁₆₅R/NP-1 or NP-2 may be produced according to the methods set forth in the Examples.

 The receptors are preferably produced by recombinant methods. A wide variety of molecular and biochemical methods are available for generating and expressing the polypeptides of the present invention; see e.g. the procedures disclosed
20 in *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), *Current Protocols in Molecular Biology* (Eds. Aufubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y. 1992) or other procedures that are otherwise known in the art. For example, the polypeptides of the invention may be obtained by chemical
25 synthesis, expression in bacteria such as *E. coli* and eukaryotes such as yeast, baculovirus, or mammalian cell-based expression systems, etc., depending on the size, nature and quantity of the polypeptide.

 The term "isolated" means that the polypeptide is removed from its original environment (e.g., the native VEGF molecule). For example, a naturally-occurring
30 polynucleotides or polypeptides present in a living animal is not isolated, but the same polynucleotides or DNA or polypeptides, separated from some or all of the coexisting

materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

5 Where it is desired to express the receptor or a fragment thereof, any suitable system can be used. The general nature of suitable vectors, expression vectors and constructions therefor will be apparent to those skilled in the art.

 Suitable expression vectors may be based on phages or plasmids, both of which are generally host-specific, although these can often be engineered for other
10 hosts. Other suitable vectors include cosmids and retroviruses, and any other vehicles, which may or may not be specific for a given system. Control sequences, such as recognition, promoter, operator, inducer, terminator and other sequences essential and/or useful in the regulation of expression, will be readily apparent to those skilled in the art.

15 Correct preparation of nucleotide sequences may be confirmed, for example, by the method of Sanger et al. (*Proc. Natl. Acad. Sci. USA* 74:5463-7 (1977)).

 A DNA fragment encoding the receptor or fragment thereof, may readily be inserted into a suitable vector. Ideally, the receiving vector has suitable restriction sites for ease of insertion, but blunt-end ligation, for example, may also be used, although
20 this may lead to uncertainty over reading frame and direction of insertion. In such an instance, it is a matter of course to test transformants for expression, 1 in 6 of which should have the correct reading frame. Suitable vectors may be selected as a matter of course by those skilled in the art according to the expression system desired.

 By transforming a suitable organism or, preferably, eukaryotic cell line, such
25 as HeLa, with the plasmid obtained, selecting the transformant with ampicillin or by other suitable means if required, and adding tryptophan or other suitable promoter-inducer (such as indoleacrylic acid) if necessary, the desired polypeptide or protein may be expressed. The extent of expression may be analyzed by SDS polyacrylamide gel electrophoresis-SDS-PAGE (Lemelli, *Nature* 227:680-685
30 (1970)).

Suitable methods for growing and transforming cultures etc. are usefully illustrated in, for example, Maniatis (Molecular Cloning, A Laboratory Notebook, Maniatis et al. (eds.), Cold Spring Harbor Labs, N.Y. (1989)).

5 Cultures useful for production of polypeptides or proteins may suitably be cultures of any living cells, and may vary from prokaryotic expression systems up to eukaryotic expression systems. One preferred prokaryotic system is that of *E. coli*, owing to its ease of manipulation. However, it is also possible to use a higher system, such as a mammalian cell line, for expression of a eukaryotic protein. Currently preferred cell lines for transient expression are the HeLa and Cos cell lines. Other
10 expression systems include the Chinese Hamster Ovary (CHO) cell line and the baculovirus system.

Other expression systems which may be employed include streptomyces, for example, and yeasts, such as *Saccharomyces* spp., especially *S. cerevisiae*. Any system may be used as desired, generally depending on what is required by the
15 operator. Suitable systems may also be used to amplify the genetic material, but it is generally convenient to use *E. coli* for this purpose when only proliferation of the DNA is required.

The polypeptides and proteins may be isolated from the fermentation or cell culture and purified using any of a variety of conventional methods including: liquid
20 chromatography such as normal or reversed phase, using HPLC, FPLC and the like; affinity chromatography (such as with inorganic ligands or monoclonal antibodies); size exclusion chromatography; immobilized metal chelate chromatography; gel electrophoresis; and the like. One of skill in the art may select the most appropriate isolation and purification techniques without departing from the scope of this
25 invention.

Additionally, the DNA encoding VEGF₁₆₅ R/NP-1 or NP-2 can be used to treat ischemia, e.g., heart and limb. The DNA can be used as an adjunct to gene therapy with VEGF (See, e.g., Isner, J., WO 97/14307). For example, the DNA can be delivered to the vicinity of the area to be treated either prior to, or along with, the
30 VEGF or DNA encoding the VEGF.

The present invention also provides binding assays using VEGF₁₆₅R/NP-1 or NP-2 that permit the ready screening for compounds which affect the binding of the receptor and its ligands, e.g., VEGF₁₆₅. These assays can be used to identify compounds that modulate, preferably inhibit metastasis. However, it is also important
5 to know if a compound enhances metastasis so that its use can be avoided. For example, in a direct binding assay the compound of interest can be added before or after the addition of the labeled ligand, e.g., VEGF₁₆₅ and the effect of the compound on binding or metastasis can be determined by comparing the degree of binding in that situation against a base line standard with that ligand, not in the presence of the
10 compound. The assay can be adapted depending upon precisely what is being tested.

The preferred technique for identifying molecules which bind to the VEGF₁₆₅R/NP-1 receptor utilizes a receptor attached to a solid phase, such as the well of an assay plate. The binding of the candidate molecules, which are optionally labeled (e.g., radiolabeled), to the immobilized receptor can be measured. Alternatively,
15 competition for binding of a known, labeled receptor ligand, such as I-¹²⁵ VEGF₁₆₅, can be measured. For screening for antagonists, the VEGF₁₆₅R/NP-1 receptor can be exposed to a receptor ligand, e.g., VEGF₁₆₅, followed by the putative antagonist, or the ligand and antagonist can be added to the VEGF₁₆₅R/NP-1 receptor simultaneously, and the ability of the antagonist to block receptor activation can be evaluated. For
20 example, VEGF antagonist activity may also be determined by inhibition of binding of labeled VEGF₁₆₅ to VEGF₁₆₅R as disclosed in the Examples.

The ability of discovered antagonists to influence angiogenesis or metastasis can also be determined using a number of know *in vivo* and *in vitro* assays. Such assays are disclosed in Jain et al., *Nature Medicine* 3, 1203-1208(1997), and the
25 examples.

The present invention further provides use of the VEGF₁₆₅R/NP-1 for intracellular or extracellular targets to affect binding. Intracellular targeting can be accomplished through the use of intracellularly expressed antibodies referred to as intrabodies. Extracellular targeting can be accomplished through the use of receptor
30 specific antibodies. Additionally, the soluble form of the receptor can be used as a receptor decoy to inhibit binding.

These methods can be used to inhibit metastasis in malignant cells as we have found that the presence of these receptors is positively correlated with metastasis. One can treat a range of afflictions or diseases associated with expression of the receptor by directly blocking the receptor. This can be accomplished by a range of different approaches. For example, antibodies, decoys, small molecules, antagonists, etc. One preferred approach is the use of antibodies that specifically block VEGF binding to the receptor. For example, an antibody to the VEGF binding site. Antibodies to these receptors can be prepared by standard means. For example, one can use single chain antibodies to target these binding sites.

One type of decoy can be obtained by deleting or otherwise changing the transmembrane portion of the molecule. The cytoplasmic tail can also be deleted in one embodiment, or retained in another.

In accordance with yet another aspect of the present invention, there are provided isolated antibodies or antibody fragments which selectively binds the receptor. The antibody fragments include, for example, Fab, Fab', F(ab')₂ or Fv fragments. The antibody may be a single chain antibody, a humanized antibody or a chimeric antibody.

Antibodies, or their equivalents, or other receptor antagonists may also be used in accordance with the present invention for the treatment or prophylaxis of cancers. Administration of a suitable dose of the antibody or the antagonist may serve to block the receptor and this may provide a crucial time window in which to treat the malignant growth.

Prophylaxis may be appropriate even at very early stages of the disease, as it is not known what specific event actually triggers metastasis in any given case. Thus, administration of the antibodies, their equivalents, intrabodies, decoys or antagonists which interfere with receptor activity, may be effected as soon as cancer is diagnosed, and treatment continued for as long as is necessary, preferably until the threat of the disease has been removed. Such treatment may also be used prophylactically in individuals at high risk for development of certain cancers, e.g., prostate or breast.

A method of treatment involves attachment of a suitable toxin to the antibodies which then target the area of the tumor. Such toxins are well known in the art, and

may comprise toxic radioisotopes, heavy metals; enzymes and complement activators, as well as such natural toxins as ricin which are capable of acting at the level of only one or two molecules per cell. It may also be possible to use such a technique to deliver localized doses of suitable physiologically active compounds, which may be used, for example, to treat cancers.

It will be appreciated that antibodies for use in accordance with the present invention, whether for diagnostic or therapeutic applications, may be monoclonal or polyclonal as appropriate. Antibody equivalents of these may comprise: the Fab' fragments of the antibodies, such as Fab, Fab', F(ab')₂ and Fv; idiotopes; or the results of allotope grafting (where the recognition region of an animal antibody is grafted into the appropriate region of a human antibody to avoid an immune response in the patient), for example. Single chain antibodies may also be used. Other suitable modifications and/or agents will be apparent to those skilled in the art.

Chimeric and humanized antibodies are also within the scope of the invention. It is expected that chimeric and humanized antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody. A variety of approaches for making chimeric antibodies, comprising for example a non-human variable region and a human constant region, have been described. See, for example, Morrison et al., Proc. Natl. Acad. Sci. U.S.A. 81,6851 (1985); Takeda, et al., Nature 314,452(1985), Cabilly et al., U.S. Pat. No. 4,816,567; Boss et al., U.S. Pat. No. 4,816,397; Tanaguchi et al., European Patent Publication EP 171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B. Additionally, a chimeric antibody can be further "humanized" such that parts of the variable regions, especially the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such altered immunoglobulin molecules may be made by any of several techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and are preferably made according to the teachings of PCT Publication WO92/06193 or EP 0239400. Humanized antibodies can be commercially produced by, for example, Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.

The antibody can be administered by a number of methods. One preferred method is set forth by Marasco and Haseltine in PCT WO94/02610, which is incorporated herein by reference. This method discloses the intracellular delivery of a gene encoding the antibody. One would preferably use a gene encoding a single chain antibody. The antibody would preferably contain a nuclear localization sequence. One preferably uses an SV40 nuclear localization signal. By this method one can intracellularly express an antibody, which can block VEGF₁₆₅R/NP-1 or NP-2 functioning in desired cells.

Where the present invention provides for the administration of, for example, antibodies to a patient, then this may be by any suitable route. If the tumor is still thought to be, or diagnosed as, localized, then an appropriate method of administration may be by injection direct to the site. Administration may also be by injection, including subcutaneous, intramuscular, intravenous and intradermal injections.

Formulations may be any that are appropriate to the route of administration, and will be apparent to those skilled in the art. The formulations may contain a suitable carrier, such as saline, and may also comprise bulking agents, other medicinal preparations, adjuvants and any other suitable pharmaceutical ingredients. Catheters are one preferred mode of administration.

VEGF₁₆₅R/NP-1 expression may also be inhibited *in vivo* by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. An antisense nucleic acid molecule which is complementary to a nucleic acid molecule encoding receptor can be designed based upon the isolated nucleic acid molecules encoding the receptor provided by the invention. An antisense nucleic acid molecule can comprise a nucleotide sequence which is complementary to a coding strand of a nucleic acid, e.g. complementary to an mRNA sequence, constructed according to the rules of Watson and Crick base pairing, and can hydrogen bond to the coding strand of the nucleic acid. The antisense sequence complementary to a sequence of an mRNA can be complementary to a sequence in the coding region of the mRNA or can be complementary to a 5' or 3' untranslated region of the mRNA. Furthermore, an

antisense nucleic acid can be complementary in-sequence to a regulatory region of the gene encoding the mRNA, for instance a transcription initiation sequence or regulatory element. Preferably, an antisense nucleic acid complementary to a region preceding or spanning the initiation codon or in the 3' untranslated region of an mRNA is used. An antisense nucleic acid can be designed based upon the nucleotide sequence shown in SEQ ID NO:1 (VEGF₁₆₅R/NP-1) or SEQ ID NO:3/NP-2). A nucleic acid is designed which has a sequence complementary to a sequence of the coding or untranslated region of the shown nucleic acid. Alternatively, an antisense nucleic acid can be designed based upon sequences of a VEGF₁₆₅R gene, which can be identified by screening a genomic DNA library with an isolated nucleic acid of the invention. For example, the sequence of an important regulatory element can be determined by standard techniques and a sequence which is antisense to the regulatory element can be designed.

The antisense nucleic acids and oligonucleotides of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The antisense nucleic acid or oligonucleotide can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids e.g. phosphorothioate derivatives and acridine substituted nucleotides can be used. Alternatively, the antisense nucleic acids and oligonucleotides can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e. nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). The antisense expression vector is introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, Reviews - Trends in Genetics, Vol. 1 (1)1986.

The term "pharmaceutically acceptable"-refers to compounds and compositions which may be administered to mammals without undue toxicity. Exemplary pharmaceutically acceptable salts include mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

The antibodies or nucleic acids of the invention are administered orally, topically, or by parenteral means, including subcutaneous and intramuscular injection, implantation of sustained release depots, intravenous injection, intranasal administration, and the like. Accordingly, antibodies or nucleic acids of the invention may be administered as a pharmaceutical composition comprising the antibody or nucleic acid of the invention in combination with a pharmaceutically acceptable carrier. Such compositions may be aqueous solutions, emulsions, creams, ointments, suspensions, gels, liposomal suspensions, and the like. Suitable carriers (excipients) include water, saline, Ringer's solution, dextrose solution, and solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, Carbopol Registered TM, vegetable oils, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents, for example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like. Cream or ointment bases useful in formulation include lanolin, Silvadene Registered TM (Marion), Aquaphor Registered TM (Duke Laboratories), and the like. Other topical formulations include aerosols, bandages, and other wound dressings. Alternatively one may incorporate or encapsulate the compounds such as a VEGF antagonist in a suitable polymer matrix or membrane, thus providing a sustained-release delivery device suitable for implantation near the site to be treated locally. Other devices include indwelling catheters and devices such as the Alzet Registered TM minipump. Ophthalmic preparations may be formulated using commercially available vehicles such as Sorbi-care Registered TM (Allergan), Neodecadron Registered TM (Merck, Sharp & Dohme), Lacrilube Registered TM, and the like, or may employ topical preparations such as that described in U.S. Pat. No. 5,124,155, incorporated herein by reference. Further, one may provide a VEGF antagonist in solid form, especially as a lyophilized powder. Lyophilized formulations

typically contain stabilizing and bulking agents; for example human serum albumin, sucrose, mannitol, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co.).

5 The amount of antibody or nucleic acid required to treat any particular disorder will of course vary depending upon the nature and severity of the disorder, the age and condition of the subject, and other factors readily determined by one of ordinary skill in the art.

All references cited above or below are herein incorporated by reference.

10 The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Experimental procedures

15 Materials

Cell culture media, lipofectin and lipofectamin reagents for transfection were purchased from Life Technologies. Human recombinant VEGF₁₆₅ and VEGF₁₂₁ were produced in Sf-21 insect cells infected with recombinant baculovirus vectors encoding
20 either human VEGF₁₆₅ or VEGF₁₂₁ as previously described (Cohen et al., *Growth Factors*, 7, 131-138 (1992); Cohen et al., *J. Biol. Chem.*, 270, 11322-11326 (1995)). GST VEGF exons 7+8 fusion protein was prepared in E.Coli and purified as previously described (Soker et al., *J. Biol. Chem.*, 271, 5761-5767 (1996)). Heparin, hygromycin B and protease inhibitors were purchased from Sigma (St. Louis,
25 MO). ¹²⁵I-Sodium, ³²P-dCTP, and GeneScreen-Plus hybridization transfer membrane were purchased from DuPont NEN (Boston, MA). Disuccinimidyl suberate (DSS) and IODO-BEADS were purchased from Pierce Chemical Co. (Rockford, IL). Con A Sepharose was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). RNAzol-B was purchased from TEL-TEST Inc. (Friendswood, TX). Silver Stain kit
30 and Trans-Blot PVDF membranes were purchased from Bio-Rad Laboratories (Hercules, CA). Multiple tissue northern blot membranes were purchased from Clontech (Palo Alto, CA). PolyA Tract mRNA isolation kits were purchased from

Promega (Madison, WI). RediPrime DNA labeling kits and molecular weight markers were purchased from Amersham (Arlington Heights, IL). Plasmids: pcDNA3.1 was purchased from Invitrogen (Carlsbad, CA), and pCPhygro, containing the CMV promoter and encoding hygromycin B phosphorylase, was kindly provided by Dr.

- 5 Urban Deutsch (Max Plank Institute, Bad Nauheim, Germany). Restriction endonucleases and Ligase were purchased from New England Biolabs, Inc (Beverly, MA). NT-B2 photographic emulsion and x-ray film were purchased from the Eastman Kodak company (Rochester NY).

10 Cell culture

- Human umbilical vein EC (HUVEC) were obtained from American Type Culture Collection (ATCC) (Rockville, MD), and grown on gelatin coated dishes in M-199 medium containing 20% fetal calf serum (FCS) and a mixture of glutamine, penicillin and streptomycin (GPS). Basic FGF (2 ng/ml) was added to the culture
- 15 medium every other day. Parental porcine aortic endothelial (PAE) cells and PAE cells expressing KDR (PAE/KDR) (Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994)) were kindly provided by Dr. Lena Claesson-Welsh and were grown in F12 medium containing 10% FCS and GPS. MDA-MB-231 cells and MDA-MB-453 cells were obtained from ATCC, and grown in DMEM containing 10% FCS and GPS. The
- 20 human melanoma cell lines, RU-mel, EP-mel and WK-mel were kindly provided by Dr. Randolph Byer (Boston University Medical School, Boston, MA), and grown in DMEM containing 2% FCS, 8% calf serum and GPS. Human metastatic prostate adenocarcinoma, LNCaP and prostate carcinoma, PC3 cells were kindly provided by Dr. Michael Freeman (Children's Hospital, Boston, MA), and grown in RPMI 1640
- 25 containing 5% FCS and GPS.

Purification and protein sequencing

- Approximately 5×10^8 MDA-MB-231 cells grown in 150 cm dishes were washed with PBS containing 5 mM EDTA, scraped and centrifuged for 5 min at 500g. The cell pellet
- 30 was lysed with 150 ml of 20 mM HEPES, pH 8.0, 0.5% triton X-100 and protease inhibitors including 1 mM AEBSF, 5 μ g/ml leupeptin and 5 μ g/ml aprotinin for 30 min on ice, and the

lysate was centrifuged at 30,000 x g for 30 min. MnCl_2 and CaCl_2 were added to the supernatant to obtain a final concentration of 1mM each. The lysate was absorbed onto a Con A Sepharose column (7 ml) and bound proteins were eluted with 15 ml 20 mM HEPES, pH 8.0, 0.2 M NaCl, 0.1% triton X-100 and 1 M methyl- α -D-mannopyranoside at 0.2 ml/min.

5 The elution was repeated twice more at 30 minute intervals. The Con A column eluates were pooled and incubated for 12 h at 4°C with 0.5 ml of VEGF₁₆₅- Sepharose beads, containing about 150 μg VEGF₁₆₅, prepared as described previously (Wilchek and Miron, *Biochem. Int.* 4, 629-635. (1982)). The VEGF₁₆₅-Sepharose beads were washed with 50 ml of 20 mM HEPES, pH 8.0, 0.2 M NaCl and 0.1% triton X-100 and then with 25 ml of 20 mM HEPES,

10 pH 8.0. The beads were boiled in SDS-PAGE buffer and bound proteins were separated by 6% SDS-PAGE. Proteins were transferred to a TransBlot PVDF membrane using a semi-dry electric blotter (Hoeffer Scientific), and the PVDF membrane was stained with 0.1% Coomassie Brilliant Blue in 40% methanol. The two prominent proteins in a 130-140 kDa doublet were cut out separately and N-terminally sequenced using an Applied Biosystems

15 model 477A microsequenator as a service provided by Dr. William Lane of the Harvard Microchemistry facility (Cambridge, MA).

Expression cloning and DNA sequencing

Complementary DNA (cDNA) was synthesized from 5 μg 231 mRNA.

20 Double-stranded cDNA was ligated to *EcoRI* adaptors, and size-fractionated on a 5-20% potassium acetate gradient. DNA fragments larger than 2kb were ligated to an eukaryotic expression plasmid, pcDNA3.1. The plasmid library was transfected into *E.coli* to yield a primary library of approximately 1×10^7 individual clones. A portion of the transformed bacteria was divided into 240 pools, each representing

25 approximately 3×10^3 individual clones. DNA prepared from each pool was used to transfect COS-7 cells seeded in 12 well dishes, using the Lipofectin reagent according to the manufacturer's instructions. Three days after transfection, the cells were incubated on ice for 2 h with ^{125}I -VEGF₁₆₅ (10 ng/ml) in the presence of 1 $\mu\text{g}/\text{ml}$ heparin, washed and fixed with 4% paraformaldehyde in PBS. ^{125}I -VEGF₁₆₅ binding to

30 individual cells was detected by overlaying the monolayers with photographic emulsion, NT-B2, and developing the emulsion after two days as described (Gearing

et al., 1989). Seven positive DNA pools were identified and DNA from one of the positive pools was used to transform *E. Coli*. The *E. coli* were sub-divided into 50 separate pools and plated onto 50 LB ampicillin dishes, with each pool representing approximately 100 clones. DNA made from these pools was transfected into COS-7 cells which were screened for ^{125}I -VEGF₁₆₅ binding as described above. Twenty positive pools were detected at this step, and their corresponding DNAs were used to transform *E. Coli*. Each pool was plated onto separate LB ampicillin dishes and DNA was prepared from 96 individual colonies and screened in a 96-well two dimensional grid for ^{125}I -VEGF₁₆₅ binding to transfected COS-7 cells as described above. Seven single clones were identified as being positive at this step. The seven positive plasmid clones were amplified and their DNA was analyzed by restriction enzyme digestion. Six clones showed an identical digestion pattern of digest and one was different. One clone from each group was submitted for automated DNA sequencing.

15 Northern Analysis

Total RNA was prepared from cells in culture using RNAzol according to the manufacturer's instructions. Samples of 20 μg RNA were separated on a 1% formaldehyde-agarose gel, and transferred to a GeneScreen-Plus membrane. The membrane was hybridized with a ^{32}P labeled fragment of human VEGF₁₆₅R/NP-1 cDNA, corresponding to nucleotides 63-454 in the ORF, at 63°C for 18 h. The membrane was washed and exposed to an x-ray film for 18 h. A commercially-obtained multiple human adult tissue mRNA blot (Clontech, 2 μg /lane) was probed for human NP-1 in a similar manner. The multiple tissue blot was stripped by boiling in the presence of 0.5% SDS and re-probed with a ^{32}P labeled fragment of KDR cDNA corresponding to nucleotides 2841-3251 of the ORF (Terman et al., *Oncogene* 6, 1677-1683 (1991)).

Transfection of PAE cells

Parental PAE cells and PAE cells expressing KDR (PAE/KDR) (Waltenberger et al., 1994) were obtained from Dr. Lena Claesson-Welsh. Human NP-1 cDNA was digested with *Xho*I and *Xba*I restriction enzymes and subcloned into the

corresponding sites of pCPhygro, to yield pCPhyg-NP-1. PAE and PAE/KDR cells were grown in 6 cm dishes and transfected with 5 μ g of pCPhyg-NP-1 using Lipofectamine, according to the manufacturer's instructions. Cells were allowed to grow for an additional 48 h and the medium was replaced with fresh medium containing 200 μ g/ml hygromycin B. After 2 weeks, isolated colonies (5-10 $\times 10^3$ cell/colony) were transferred to separate wells of a 48 well dish and grown in the presence of 200 μ g/ml hygromycin B. Stable PAE cell clones expressing VEGF₁₆₅R/NP-1 (PAE/NP-1) or co-expressing VEGF₁₆₅R/NP-1 and KDR (PAE/KDR/NP-1) were screened for VEGF₁₆₅ receptor expression by binding and cross linking of ¹²⁵I-VEGF₁₆₅. For transient transfection, PAE/KDR cells were transfected with VEGF₁₆₅R/NP-1 as described above and after three days ¹²⁵I-VEGF₁₆₅ cross-linking analysis was carried out.

Radio-iodination of VEGF, binding and cross-linking experiments.

The radio-iodination of VEGF₁₆₅ and VEGF₁₂₁ using IODO-BEADS was carried out as previously described (Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)). The specific activity ranged from 40,000-100,000 cpm/ng protein. Binding and cross-linking experiments using ¹²⁵I-VEGF₁₆₅ and ¹²⁵I-VEGF₁₂₁ were performed as previously described (Gitay-Goren et al., *J. Biol. Chem.* 267, 6093-6098 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). VEGF binding was quantitated by measuring the cell-associated radioactivity in a γ -counter (Beckman, Gamma 5500). The counts represent the average of three wells. All experiments were repeated at least three times and similar results were obtained. The results of the binding experiments were analyzed by the method of Scatchard using the LIGAND program (Munson and Rodbard, 1980). ¹²⁵I-VEGF₁₆₅ and ¹²⁵I-VEGF₁₂₁ cross linked complexes were resolved by 6% SDS/PAGE and the gels were exposed to an X-Ray film. X-ray films were subsequently scanned by using an IS-1000 digital imaging system (Alpha Innotech Corporation)

Purification of VEGF₁₆₅R

Cross-linking of ¹²⁵I-VEGF₁₆₅ to cell surface receptors of 231 cells results in formation of a 165-175 kDa labeled complex (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). These cells have about 1-2 x 10⁵ VEGF₁₆₅ binding sites/cell. In contrast to VEGF₁₆₅, VEGF₁₂₁ does not bind to the 231 cells and does not form a ligand-receptor complex (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). The relatively high VEGF₁₆₅R number and the lack of any detectable KDR or Flt-1 mRNA in 231 cells (not shown) suggested that these cells would be a useful source for VEGF₁₆₅R purification. Preliminary characterization indicated that VEGF₁₆₅R is a glycoprotein and accordingly, a 231 cell lysate prepared from approximately 5 x 10⁸ cells was absorbed onto a Con A Sepharose column. Bound proteins, eluted from the Con A column, were incubated with VEGF₁₆₅-Sepharose and the VEGF₁₆₅- affinity purified proteins were analyzed by SDS-PAGE and silver staining (Figure 9, lane 2). A prominent doublet with a molecular mass of about 130-135 kDa was detected. This size is consistent with the formation of a 165-175 kDa complex of 40-45 kDa VEGF₁₆₅ bound to receptors approximately 130-135 kDa in size (Figure 9, lane 1). The two bands were excised separately and N-terminal amino acid sequencing was carried out (Figure 1, right). Both the upper and lower bands had similar N-terminal amino acid sequences which showed high degrees of sequence homology to the predicted amino acid sequences in the N-terminal regions of mouse (Kawakami et al., *J. Neurobiol.*, 29, 1-17 (1995)) and human neuropilin-1 (NP-1) (He and Tessier-Lavigne, *Cell* 90739-751 (1997)).

Expression cloning of VEGF₁₆₅R from 231 cell-derived mRNA

Concomitant with the purification, VEGF₁₆₅R was cloned by expression cloning (Aruffo and Seed, *Proc. Natl. Acad. Sci. USA* 84, 8573-8577 (1987a); Aruffo and Seed, *EMBO J.*, 6, 3313-3316 (1987b); Gearing et al., *EMBO J.* 8,3667-3676 (1989)). For expression cloning, 231 cell mRNA was used to prepare a cDNA library of approximately 10⁷ clones in a eukaryotic expression plasmid. *E. coli* transformed with the plasmid library were divided into pools. The DNA prepared from each pool were transfected into COS-7 cells in separate wells and individual cells were screened

for the ability to bind ^{125}I -VEGF₁₆₅ as detected by autoradiography of monolayers overlaid with photographic emulsion (Fig 2A). After three rounds of subpooling and screening, seven single positive cDNA clones were obtained. Figure 2B shows binding of ^{125}I -VEGF₁₆₅ to COS-7 cells transfected with one of these single positive clones (clone A2).

Restriction enzyme analysis revealed that six of the seven positive single clones had identical restriction digestion patterns but that one clone had a pattern that was different (not shown). Sequencing of one of these similar cDNA clones, clone A2 (Figure 3), showed it to be identical to a sequence derived from a human-expressed sequence tag data bank (dbEST). This sequence also showed a high percentage of homology to the sequence of mouse neuropilin, NP-1 (Kawakami et al., *J. Neurobiol* 29, 1-17 (1995)). After we had cloned human VEGF₁₆₅R, two groups reported the cloning of rat and human receptors for semaphorin III and identified them to be NP-1 (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)). The 231 cell-derived VEGF₁₆₅R cDNA sequence is virtually identical (see figure legend 3 for exceptions) to the human NP-1 sequence (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997)). Significantly, the predicted amino acid sequence obtained by expression cloning (Figure 3) confirmed the identification of VEGF₁₆₅R as NP-1 that was determined by N-terminal sequencing (Figure 1), and we have therefore named this VEGF receptor, VEGF₁₆₅R/NP-1.

The human VEGF₁₆₅R/NP-1 cDNA sequence predicts an open reading frame (ORF) of 923 amino acids with two hydrophobic regions representing putative signal peptide and transmembrane domains (Figure 3). Overall, the sequence predicts ectodomain, transmembrane and cytoplasmic domains consistent with the structure of a cell surface receptor. The N-terminal sequence obtained via protein purification as shown in Figure 1 is downstream of a 21 amino acid putative hydrophobic signal peptide domain, thereby indicating directly where the signal peptide domain is cleaved and removed. The short cytoplasmic tail of 40 amino acids is consistent with results demonstrating that soluble VEGF₁₆₅R/NP-1 released by partial trypsin digestion of 231 cells is similar in size to intact VEGF₁₆₅R/NP-1 (not shown).

Sequence analysis of the one clone obtained by expression cloning that had a different restriction enzyme profile predicted an open reading frame of 931 amino acids with about a 47% homology to VEGF₁₆₅R/NP-1 (Figure 4). This human cDNA has a 93% sequence homology with rat neuropilin-2 (NP-2) and is identical to the recently cloned human NP-2 (Chen et al., *Neuron*, 19, 547-559 (1997)).

Expression of VEGF₁₆₅R/NP-1 in adult cell lines and tissues

Reports of NP-1 gene expression have been limited so far to the nervous system of the developing embryo (Takagi et al., *Dev. Biol.* 122, 90-100 (1987); Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995); Takagi et al., *Dev. Biol.* 170, 207-222 (1995)). Cell surface VEGF₁₆₅R/NP-1, however, is associated with non-neuronal adult cell types such as EC and a variety of tumor-derived cells (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). Northern blot analysis was carried out to determine whether cells that crossed-linked ¹²⁵I-VEGF₁₆₅ also synthesized VEGF₁₆₅R/NP-1 mRNA. (Figure 5). VEGF₁₆₅R/NP-1 mRNA levels were highest in 231 and PC3 cells. VEGF₁₆₅R/NP-1 mRNA was detected to a lesser degree in HUVEC, LNCaP, EP-mel and RU-mel cells. There was little if any expression in MDA-MB-453 and WK-mel cells. The VEGF₁₆₅R/NP-1 gene expression patterns were consistent with our previous results showing that HUVEC, 231, PC3, LNCaP, EP-mel and RU-mel cells bind ¹²⁵I-VEGF₁₆₅ to cell surface VEGF₁₆₅R/NP-1 but that MDA-MB-453 and WK-mel cells do not (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)).

VEGF₁₆₅R/NP-1 gene expression was analyzed also by Northern blot in a variety of adult tissues in comparison to KDR gene expression (Figure 6). VEGF₁₆₅R/NP-1 mRNA levels were relatively highly in adult heart and placenta and relatively moderate in lung, liver, skeletal muscle, kidney and pancreas. A relatively low level of VEGF₁₆₅R/NP-1 mRNA was detected in adult brain. Interestingly, previous analysis of NP-1 gene expression in mouse and chicken brain suggested that this gene was expressed primarily during embryonic development and was greatly diminished after birth (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995); Takagi et al., *Dev. Biol.* 170, 207-222 (1995)). The tissue distribution of KDR mRNA was similar to that of VEGF₁₆₅R/NP-1 with the exception that it was not expressed highly in the

heart. These results indicate that VEGF₁₆₅R/NP-1 is expressed widely in adult non-neuronal tissue, including tissues in which angiogenesis occurs such as heart and placenta.

5 Characterization of VEGF₁₆₅ binding to VEGF₁₆₅R/NP-1

In order to characterize the binding properties of VEGF₁₆₅R/NP-1, porcine aortic endothelial (PAE) cells were transfected with the cDNA of VEGF₁₆₅R/NP-1. The PAE cells were chosen for these expression studies because they express neither KDR, Flt-1 (Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994)) nor VEGF₁₆₅R. Stable cell lines synthesizing VEGF₁₆₅R/NP-1 (PAE/NP-1) were established and ¹²⁵I-VEGF₁₆₅ binding experiments were carried out (Fig 7). ¹²⁵I-VEGF₁₆₅ binding to PAE/NP-1 cells increased in a dose-dependent manner and reached saturation at approximately 30 ng/ml demonstrating that VEGF₁₆₅R/NP-1 is a specific VEGF₁₆₅ receptor (Figure 7A). Scatchard analysis of VEGF₁₆₅ binding revealed a single class of VEGF₁₆₅ binding sites with a K_d of approximately 3.2 x 10⁻¹⁰ M and approximately 3 x 10⁵ ¹²⁵I-VEGF₁₆₅ binding sites per cell (Figure 7B). Similar K_d values were obtained for several independently-generated PAE/NP-1 clones, although the receptor number varied from clone to clone (not shown). The K_d of 3 x 10⁻¹⁰ M for the PAE/NP-1 cell lines is consistent with the 2-2.8 x 10⁻¹⁰ M K_d values obtained for VEGF₁₆₅R/NP-1 expressed naturally by HUVEC and 231 cells (Gitay-Goren et al., *J. Biol. Chem.* 267, 6093-6098 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). The binding of ¹²⁵I-VEGF₁₆₅ to PAE/NP-1 cells was enhanced by 1 µg/ml heparin (not shown), consistent with previous studies showing that heparin enhances ¹²⁵I-VEGF₁₆₅ binding to VEGF₁₆₅R/NP-1 on HUVEC and 231 cells (Gitay-Goren et al., *J. Biol. Chem.* 267, 6093-6098 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)).

Isoform-specific binding of VEGF to cells expressing VEGF₁₆₅R/NP-1

VEGF₁₆₅, but not VEGF₁₂₁, binds to VEGF₁₆₅R/NP-1 on HUVEC and 231 cells (Gitay-Goren et al., *J. Biol. Chem.* 271, 5519-5523 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). To ascertain whether cells transfected with

VEGF₁₆₅R/NP-1 had the same binding specificity, PAE/NP-1 cells were incubated with ¹²⁵I-VEGF₁₆₅ or ¹²⁵I-VEGF₁₂₁ followed by cross-linking (Figure 8). ¹²⁵I-VEGF₁₆₅ did not bind to parental PAE cells (Figure 8, lane 3) but did bind to PAE/NP-1 cells via VEGF₁₆₅R/NP-1 (Figure 8, lane 4). The radiolabeled complexes formed with VEGF₁₆₅R/NP-1 were similar in size to those formed in HUVEC (Figure 8, lane 1) and PC3 cells (Figure 8, lane 2). On the other hand, ¹²⁵I-VEGF₁₂₁ did not bind to either parental PAE (Figure 8, lane 7) or to PAE/NP-1 cells (Figure 8, lane 8). These results demonstrate that the VEGF isoform-specific binding that occurs with cells expressing endogenous VEGF₁₆₅R/NP-1 such as HUVEC, 231 and PC3 cells, can be replicated in cells transfected with VEGF₁₆₅R/NP-1 cDNA and support the finding that VEGF₁₆₅R and NP-1 are identical.

Co-expression of VEGF₁₆₅R/NP-1 and KDR modulates VEGF₁₆₅ binding to KDR

To determine whether expression of VEGF₁₆₅R/NP-1 had any effect on VEGF₁₆₅ interactions with KDR, PAE cells that were previously transfected with KDR cDNA to produce stable clones of PAE/KDR cells (Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994)), were transfected with VEGF₁₆₅R/NP-1 cDNA and stable clones expressing both receptors (PAE/KDR/NP-1) were obtained. These cells bound ¹²⁵I-VEGF₁₆₅ to KDR (Figure 8, lane 6, upper complex) and to VEGF₁₆₅R/NP-1 (Figure 8, lane 6, lower complex) to yield a cross-linking profile similar to HUVEC (Figure 8, lane 1). On the other hand, the PAE/KDR/NP-1 cells bound ¹²⁵I-VEGF₁₂₁ to form a complex only with KDR (Figure 8, lanes 9 and 10), consistent with the inability of VEGF₁₂₁ to bind VEGF₁₆₅R/NP-1.

It appeared that in cells co-expressing KDR and VEGF₁₆₅R/NP-1 (Figure 8, lane 6), the degree of ¹²⁵I-VEGF₁₆₅-KDR 240 kDa complex formation was enhanced compared to the parental PAE/KDR cells (Figure 8, lane 5). These results were reproducible and the degree of ¹²⁵I-VEGF₁₆₅-KDR 240 kDa complex formation in different clones was correlated positively with the levels of VEGF₁₆₅R/NP-1 expressed (not shown). However, it could not be ruled out definitively that these differential KDR binding results were possibly due to clonal selection post-transfection.

Therefore, parental PAE/KDR cells were transfected with VEGF₁₆₅R/NP-1 cDNA and ¹²⁵I-VEGF₁₆₅ was bound and cross-linked to the cells three days later in order to avoid any diversity of KDR expression among individual clones (Figure 9). A labeled 240 kDa complex containing KDR was formed in parental PAE/KDR cells (Fig 9, lane 1) and in PAE/KDR cells transfected with the expression vector (Figure 9, lane 2). However, when ¹²⁵I-VEGF₁₆₅ was cross-linked to PAE/KDR cells transiently expressing VEGF₁₆₅R/NP-1, a more intensely labeled 240 kDa complex, about 4 times greater, was observed (Figure 9, lane 3), compared to parental PAE/KDR cells (Figure 9, lane 1) and PAE/KDR cells transfected with expression vector (Figure 9, lane 2). These results suggest that co-expression of KDR and VEGF₁₆₅R/NP-1 genes in the same cell enhances the ability of VEGF₁₆₅ to bind to KDR.

A GST-VEGF Exon 7+8 fusion protein inhibits VEGF₁₆₅ binding to VEGF₁₆₅R/NP-1 and KDR

We have shown that ¹²⁵I-VEGF₁₆₅ binds to VEGF₁₆₅R/NP-1 through its exon 7-encoded domain (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). In addition, a GST fusion protein containing the peptide encoded by VEGF exon 7+8 (GST-Ex 7+8), inhibits completely the binding of ¹²⁵I-VEGF₁₆₅ to VEGF₁₆₅R/NP-1 associated with 231 cells and HUVEC (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996); Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)). When, added to PAE/NP-1 cells, the fusion protein completely inhibited binding to VEGF₁₆₅R/NP-1 (Figure 10, lane 2 compared to lane 1). On the other hand, it did not inhibit ¹²⁵I-VEGF₁₆₅ binding at all to KDR (Figure 10, lane 4 compared to lane 3). Thus, these results demonstrate that GST-Ex 7+8 binds directly to VEGF₁₆₅R/NP-1 but does not bind to KDR. The effects of GST-Ex 7+8 are different, however, in cells co-expressing both VEGF₁₆₅R/NP-1 and KDR (PAE/KDR/NP-1). Consistent with the results in Figures 8 and 9, the degree of ¹²⁵I-VEGF₁₆₅ binding to KDR in PAE/KDR/NP-1 cells (Figure 10, lane 5) was greater than to the parental PAE/KDR cells (Figure 10, lane 3). Interestingly, in PAE/KDR/NP-1 cells, GST-Ex 7+8 inhibited not only ¹²⁵I-VEGF₁₆₅ binding to VEGF₁₆₅R/NP-1 completely as expected, but it also inhibited binding to KDR substantially which was unexpected (Figure 10, lane 6 compared to lane 5). In the

presence of GST-Ex 7+8, binding of ^{125}I -VEGF₁₆₅ to KDR in these cells was reduced to the levels seen in parental PAE/KDR cells not expressing VEGF₁₆₅R/NP-1 (Figure 10, lane 6 compared to lanes 3 and 4). Since the fusion protein does not bind directly to KDR, these results suggest that inhibiting the binding of ^{125}I -VEGF₁₆₅ to

- 5 VEGF₁₆₅R/NP-1 directly, inhibits its binding to KDR indirectly. Taken together, the results in Figures 8, 9 and 10 suggest that interactions of VEGF₁₆₅ with VEGF₁₆₅R/NP-1 enhance VEGF interactions with KDR.

Neuropilin-1 is an isoform-specific VEGF₁₆₅ receptor

- 10 Recently, we described a novel 130-135 kDa VEGF cell surface receptor that binds VEGF₁₆₅ but not VEGF₁₂₁ and that we named, accordingly, VEGF₁₆₅R (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). We have now purified VEGF₁₆₅R, expression cloned its cDNA, and shown it to be identical to human neuropilin-1 (NP-1) (He and Tessier-Lavigne, *Cell* 90 739-751 (1997)). The evidence that VEGF₁₆₅R is
- 15 identical to NP-1 and that NP-1 serves as a receptor for VEGF₁₆₅ is as follows: i) purification of VEGF₁₆₅R protein from human MDA-MB-231 (231) cells using VEGF affinity, yielded a 130-140 kDa doublet upon SDS-PAGE and silver stain. N-terminal sequencing of both proteins yielded the same N-terminal sequence of 18 amino acids that demonstrated a high degree of homology to mouse NP-1 (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995)); ii) After we purified VEGF₁₆₅R from human 231 cells, the cloning of human NP-1 was reported (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997)) and the N-terminal sequence of human VEGF₁₆₅R was found to be identical to a sequence in the N-terminal region of human NP-1; iii) Expression cloning using a
- 25 231 cell cDNA library resulted in isolation of several cDNA clones and their sequences were identical to the human NP-1 cDNA sequence (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997)). The combination of purification and expression cloning has the advantage over previous studies where only expression cloning was used (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)), in allowing unambiguous identification of the NP-1 protein N-terminus;
- 30 iv) Northern blot analysis of NP-1 gene expression was consistent with previous ^{125}I -VEGF₁₆₅ cross-linking experiments (Soker et al., *J. Biol. Chem.* 271, 5761-5767

(1996)). Cells that bound VEGF₁₆₅ to VEGF₁₆₅R synthesized relatively abundant NP-1 mRNA while cells that showed very little if any VEGF₁₆₅ binding, did not synthesize much if any NP-1 mRNA; v) when NP-1 was expressed in PAE cells, the transfected, but not the parental cells, were able to bind VEGF₁₆₅ but not VEGF₁₂₁,
5 consistent with the isoform specificity of binding previously shown for HUVEC and 231 cells (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). Furthermore, the K_d of ¹²⁵I-VEGF₁₆₅ binding to PAE expressing NP-1 was about 3×10^{-10} M, consistent with previous K_d binding values of $2-2.8 \times 10^{-10}$ M for 231 cells and HUVEC (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)); and vi) The binding of VEGF₁₆₅ to cells
10 expressing NP-1 post-transfection was more efficient in the presence of heparin as was the binding of this ligand to HUVEC and 231 cells (Gitay-Goren et al., *J. Biol. Chem.* 267, 6093-6098 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). Taken together, these results show not only that VEGF₁₆₅R is identical to NP-1 but that it is a functional receptor that binds VEGF₁₆₅ in an isoform-specific manner. Accordingly,
15 we have named this VEGF receptor VEGF₁₆₅R/NP-1.

In addition to the expression cloning of VEGF₁₆₅R/NP-1 cDNA, another human cDNA clone was isolated whose predicted amino acid sequence was 47% homologous to that of VEGF₁₆₅R/NP-1 and over 90% homologous to rat neuropilin-2 (NP-2) which was recently cloned (Kolodkin et al., *Cell* 90, 753-762 (1997)). NP-2
20 binds members of the collapsin/semaphorin family selectively (Chen et al., *Neuron* 19, 547-559 (1997)).

The discovery that NP-1 serves as a receptor for VEGF₁₆₅ was a surprise since NP-1 had previously been shown to be associated solely with the nervous system during embryonic development (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995);
25 Takagi et al., *Dev. Biol.* 170, 207-222 (1995)) and more recently as a receptor for members of the collapsin/semaphorin family (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)). NP-1 is a 130-140 kDa transmembrane glycoprotein first identified in the developing *Xenopus* optic system (Takagi et al., *Dev. Biol.* 122, 90-100 (1987); Takagi et al., *Neuron* 7, 295-307
30 (1991)). NP-1 expression in the nervous system is highly regulated spatially and temporally during development and in particular is associated with those

developmental stages when axons are actively growing to form neuronal connections.(Fujisawa et al., *Dev. Neurosci.* 17, 343-349 (1995); Kawakami et al., *J. Neurobiol* 29, 1-17 (1995); Takagi et al., *Dev. Biol.* 170, 207-222 (1995)). The NP-1 protein is associated with neuronal axons but not the stomata (Kawakami et al., *J. Neurobiol* 29, 1-17 (1995)). Functionally, neuropilin has been shown to promote neurite outgrowth of optic nerve fibers in vitro (Hirata et al., *Neurosci. Res.* 17, 159-169 (1993)) and to promote cell adhesiveness (Tagaki et al., *Dev. Biol.* 170, 207-222 (1995)). Targeted disruption of NP-1 results in severe abnormalities in the trajectory of efferent fibers of the peripheral nervous system (Kitsukawa et al., *Neuron* 19, 995-1005 (1997)). Based on the these studies, it has been suggested that NP-1 is a neuronal cell recognition molecule that plays a role in axon growth and guidance (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995); He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kitsukawa et al., *Neuron* 19, 995-1005 1997; Kolodkin et al., *Cell* 90, 753-762 (1997)).

Our results are the first to show that VEGF₁₆₅R/NP-1 is also expressed in adult tissues, in contrast to the earlier studies that have shown that NP-1 expression in *Xenopus*, chicken and mouse is limited to the developmental and early post-natal stages (Fujisawa et al., *Dev. Neurosci.* 17, 343-349 (1995); Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995); Takagi et al., *Dev. Biol.* 170, 207-222 (1995)). For example, in mice, NP-1 is expressed in the developing nervous system starting in the dorsal root ganglia at day 9 and ceases at day 15 (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995)). Our Northern blot analysis of human adult tissue demonstrates relatively high levels of VEGF₁₆₅R/NP-1 mRNA transcripts in heart, placenta, lung, liver, skeletal muscle, kidney and pancreas. Interestingly, there is very little relative expression in adult brain, consistent with the mouse nervous system expression studies (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995)). VEGF₁₆₅R/NP-1 is also expressed in a number of cultured non-neuronal cell lines including EC and a variety of tumor-derived cells. A possible function of VEGF₁₆₅R/NP-1 in these cells is to mediate angiogenesis as will be discussed below.

In addition, NP-1 has been identified as a receptor for the collapsin/semaphorin family by expression cloning of a cDNA library obtained from

rat E14 spinal cord and dorsal root ganglion (DRG) tissue (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)). The collapsin/semaphorins (collapsin-D-1/Sema III/Sem D) comprise a large family of transmembrane and secreted glycoproteins that function in repulsive growth cone and axon guidance (Kolodkin et al., *Cell* 75, 1389-1399 (1993)). The repulsive effect of sema III for DRG cells was blocked by anti-NP-1 antibodies (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)). The K_d of sema III binding to NP-1, $0.15-3.25 \times 10^{-10}$ M (He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kolodkin et al., *Cell* 90, 753-762 (1997)) is similar to that of VEGF₁₆₅ binding VEGF₁₆₅/NP-1, which is about 3×10^{-10} M. These results indicate that two structurally different ligands with markedly different biological activities, VEGF-induced stimulation of EC migration and proliferation on one hand, and sema III-induced chemorepulsion of neuronal cells, on the other hand, bind to the same receptor and with similar affinity. An interesting question is whether the two ligands bind to the same site on VEGF₁₆₅R/NP-1 or to different sites. VEGF₁₆₅R/NP-1 has five discrete domains in its ectodomain, and it has been suggested that this diversity of protein modules in NP-1 is consistent with the possibility of multiple binding ligands for NP-1 (Takagi et al., *Neuron* 7, 295-307 (1991); Feiner et al., *Neuron* 19 539-545 (1997); He and Tessier-Lavigne, *Cell* 90 739-751 (1997)). Preliminary analysis does not indicate any large degree of sequence homology between sema III and VEGF exon 7 which is responsible for VEGF binding to VEGF₁₆₅R/NP-1 (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). However there may be some 3-dimensional structural similarities between the two ligands. Since both neurons and blood vessels display branching and directional migration, the question also arises as to whether VEGF₁₆₅ displays any neuronal guidance activity and whether sema III has any EC growth factor activity. These possibilities have not been examined yet. However, it may be that VEGF requires two receptors, KDR and NP-1 for optimal EC growth factor activity (Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)) and that sema III requires NP-1 and an as yet undetermined high affinity receptor for optimal chemorepulsive activity (Feiner et al., *Neuron* 19, 539-545 (1997); He and Tessier-Lavigne, *Cell* 90, 739-751 (1997); Kitsukawa et al., *Neuron* 19, 995-1005 (1997)), so

that the presence of NP-1 alone might not be sufficient for these ligands to display novel biological activities. Future studies will determine whether there are any connections between the mechanisms that regulate neurogenesis and angiogenesis.

5 VEGF₁₆₅R/NP-1 role in angiogenesis

VEGF₁₆₅R/NP-1 modulates the binding of VEGF₁₆₅ to KDR, a high affinity RTK that is an important regulator of angiogenesis as evidenced by KDR knock out experiments in mice (Shalaby et al., *Nature* 376, 62-66 (1995)). The affinity of KDR for VEGF₁₆₅ is about 50 times greater than for VEGF₁₆₅R/NP-1 (Gitay-Goren et al., *J. Biol. Chem.* 287, 6003-6096 (1992); Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994)). When VEGF₁₆₅R/NP-1 and KDR are co-expressed, the binding of ¹²⁵I-VEGF₁₆₅ to KDR is enhanced by about 4-fold compared to cells expressing KDR alone. The enhanced binding can be demonstrated in stable clones co-expressing VEGF₁₆₅R/NP-1 and KDR (PAE/KDR/NP-1 cells), and also in PAE/KDR cells
15 transfected transiently with VEGF₁₆₅R/NP-1 cDNA where clonal selection does not take place. Conversely, when the binding of ¹²⁵I-VEGF₁₆₅ to VEGF₁₆₅R/NP-1 in PAE/KDR/NP-1 cells is inhibited completely by a GST fusion protein containing VEGF exons 7+8 (GST-Ex 7+8), the binding to KDR is inhibited substantially, down to the levels observed in cells expressing KDR alone. The fusion protein binds to
20 VEGF₁₆₅R/NP-1 directly but is incapable of binding to KDR directly (Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)). Although, not wishing to be bound by theory, we believe that VEGF₁₆₅ binds to VEGF₁₆₅R/NP-1 via the exon 7-encoded domain and facilitates VEGF₁₆₅ binding to KDR via the exon 4-encoded domain (Figure 11). VEGF₁₆₅R/NP-1, with its relatively high receptor/cell number, about $0.2-2 \times 10^5$
25 (Gitay-Goren et al., *J. Biol. Chem.* 287, 6003-6096 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)), appears to serve to concentrate VEGF₁₆₅ on the cell surface, thereby providing greater access of VEGF₁₆₅ to KDR. Alternatively, binding to VEGF₁₆₅R/NP-1, VEGF₁₆₅ undergoes a conformational change that enhances its binding to KDR. The end result would be elevated KDR signaling and increased
30 VEGF activity. Although we can demonstrate enhanced binding to KDR, to date we have not been able to demonstrate enhanced VEGF mitogenicity for PAE/KDR/NP-1

cells compared to PAE/KDR cells. One reason is that these cell lines do not proliferate readily in response to VEGF as do HUVEC (Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994). Nevertheless, we have shown that VEGF₁₆₅, which binds to both KDR and VEGF₁₆₅R/NP-1, is a better mitogen for HUVEC than is VEGF₁₂₁, which
5 binds only to KDR (Keyt et al., *J. Biol. Chem.* 271, 5638-5646 (1996b); Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997). Furthermore, inhibiting VEGF₁₆₅ binding to VEGF₁₆₅R/NP-1 on HUVEC by GST-EX 7+8, inhibits binding to KDR and also inhibits VEGF₁₆₅-induced HUVEC proliferation, down to the level induced by VEGF₁₂₁ (Soker et al., *J. Biol. Chem.* 272, 31582-31588 (1997)). Taken together,
10 these results suggest a role for VEGF₁₆₅R/NP-1 in mediating VEGF₁₆₅, but not VEGF₁₂₁ mitogenic activity. The concept that dual receptors regulate growth factor binding and activity has been previously demonstrated for TGF- β , bFGF and NGF (Lopez-Casillas et al., *Cell* 67, 785-795 (1991); Yayon et al., *Cell* 64, 841-848 (1991; Barbacid, *Curr. Opin. Cell Biol.* 7, 148-155 (1995)).

15 Another connection between VEGF₁₆₅R/NP-1 and angiogenesis comes from studies in which NP-1 was overexpressed ectopically in transgenic mice (Kitsuskawa et al., *Develop.* 121, 4309-4318 (1995)). NP-1 overexpression resulted in embryonic lethality and the mice died *in utero* no later than on embryonic day 15.5 and those that survived the best had lower levels of NP-1 expression. Mice overexpressing NP-1
20 displayed morphologic abnormalities in a limited number of non-neural tissues such as blood vessels, the heart and the limbs. NP-1 was expressed in both the EC and in the mesenchymal cells surrounding the EC. The embryos possessed excess and abnormal capillaries and blood vessels compared to normal counterparts and in some cases dilated blood vessels as well. Some of the chimeric mice showed hemorrhaging,
25 mainly in the head and neck. These results are consistent with the possibility that ectopic overexpression of VEGF₁₆₅R/NP-1 results in inappropriate VEGF₁₆₅ activity, thereby mediating enhanced and/or aberrant angiogenesis. Another piece of evidence for a link between NP-1 and angiogenesis comes from a recent report showing that in mice targeted for disruption of the NP-1 gene, the embryos have severe abnormalities
30 in the peripheral nervous system but that their death *in utero* at days 10.5-12.5 is most

probably due to anomalies in the cardiovascular system (Kitsukawa et al., *Neuron* 19, 995-1005 (1997)).

VEGF₁₆₅R/NP-1 is associated with tumor-derived cells

5 The greatest degree of VEGF₁₆₅R/NP-1 expression that we have detected so far occurs in tumor-derived cells such as 231 breast carcinoma cells and PC3 prostate carcinoma cells, far more than occurs in HUVEC. The tumor cells express abundant levels of VEGF₁₆₅R/NP-1 mRNA and about 200,000 VEGF₁₆₅ receptors/cell (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). On the other hand, these tumor cells do not
10 express KDR or Flt-1 so that VEGF₁₆₅R/NP-1 is the only VEGF receptor associated with these cells. The tumor cells are therefore useful for testing whether VEGF₁₆₅R/NP-1 is a functional receptor for VEGF₁₆₅ in the absence of a KDR background. To date, we have not been able to show that VEGF₁₆₅R/NP-1 mediates a VEGF₁₆₅ signal in tumor-derived cells as measured by receptor tyrosine
15 phosphorylation. Nevertheless, VEGF₁₆₅ might have an effect on tumor cells by inducing some, as yet undetermined activity such as enhanced survival, differentiation, or motility. A recent report has demonstrated that glioma cells express a 190 kDa protein that binds VEGF₁₆₅ but not VEGF₁₂₁ efficiently (Omura et al., *J. Biol. Chem.* 272, 23317-23322 (1997)). No stimulation of tyrosine phosphorylation could be
20 demonstrated upon binding of VEGF₁₆₅ to this receptor. Whether the 190 kDa isoform-specific receptor is related to VEGF₁₆₅R/NP-1 is not known presently.

 VEGF₁₆₅R/NP-1 may have a storage and sequestration function for VEGF₁₆₅. One might envision that VEGF₁₆₅ is produced by a tumor cell and binds to VEGF₁₆₅R/NP-1 on that cell via the exon 7-encoded domain (Soker et al., *J. Biol.*
25 *Chem.* 271, 5761-5767 (1996)). The stored VEGF₁₆₅ could be then released to stimulate tumor angiogenesis in a paracrine manner. Alternatively, VEGF₁₆₅R/NP-1 may mediate a juxtacrine effect in which VEGF₁₆₅ is bound to VEGF₁₆₅R/NP-1 on a tumor cell via the exon 7-encoded domain and is also bound to KDR on a neighboring EC via the exon 4-encoded domain (Keyt et al., *J. Biol. Chem.* 271, 5638-5646
30 (1996b)). Such a mechanism could result in a more efficient way for tumor cells to attract EC, thereby enhancing tumor angiogenesis.

In summary, we have demonstrated by independent purification and expression cloning methods that the VEGF isoform specific receptor, VEGF₁₆₅R, is identical to NP-1, a cell surface protein previously identified as playing a role in embryonic development of the nervous system and as being a receptor for the
5 collapsins/semaphorins. Furthermore, binding to VEGF₁₆₅R/NP-1 enhances the binding of VEGF₁₆₅ to KDR on EC and tumor cells.

Experimental Rationale

We have discovered that tumor cell neuropilin-1 mediates tumor cell motility
10 and thereby metastasis. In a Boyden chamber motility assay, VEGF₁₆₅ (50 ng/ml) stimulates 231 breast carcinoma cell motility in a dose-response manner, with a maximal 2-fold stimulation (Fig. 15A). On the other hand, VEGF₁₂₁ has no effect on motility of these cells (Fig. 15B). Since 231 cells do not express KDR or Flt-1, these results suggest that tumor cells are directly responsive to VEGF₁₆₅ and that VEGF₁₆₅
15 might signal tumor cells via neuropilin-1. Possible candidates for mediating VEGF₁₆₅-induced motility of carcinoma cells are PI3-kinase (PI3-K) (Carpenter, et al. (1996) *Curr. Opin. Cell Biol.* 8: 153-158.). Since 231 cells do not express KDR or Flt-1, these results suggest that tumor cells are directly responsive to VEGF₁₆₅ and that VEGF₁₆₅ might signal tumor cells via neuropilin-1.

20 The other type of evidence is that neuropilin-1 expression might be associated with tumor cell motility. We have analyzed two variants of Dunning rat prostate carcinoma cells, AT2.1 cells, which are of low motility and low metastatic potential, and AT3.1 cells, which are highly motile, and metastatic. Cross-linking and Northern blot analysis show that AT3.1 cells express abundant neuropilin-1, capable of binding
25 VEGF₁₆₅, while AT2.1 cells don't express neuropilin-1 (Fig. 16). Immunostaining of tumor sections confirms the expression of neuropilin-1 in AT3.1, but not AT2.1 tumors (Fig. 17). Furthermore, the immunostaining shows that in subcutaneous AT3.1 and PC3 tumors, the tumor cells expressing neuropilin-1 are found preferentially at the invading front of the tumor/dermis boundary (Fig. 17). To determine more directly
30 whether neuropilin-1 expression is correlated with enhanced motility, neuropilin-1 was overexpressed in AT2.1 cells (Fig. 18). Three stable clones of AT2.1 cells overexpressing neuropilin-1 had enhanced motility in the Boyden chamber assay.

These results indicate that expression of neuropilin-1 in AT2.1 cells enhances their motility. Taken together, it appears that neuropilin-1 expression on tumor cells is associated with the motile, metastatic phenotype.

5 The references cited throughout the specification are incorporated herein by reference.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

What is claimed:

1. A method of diagnosing cancer in a patient, comprising: obtaining a biological specimen; measuring levels of a neutrophilin in said specimen, wherein levels of neutrophilin in said sample greater than a base line level is indicative of cancer.
2. The method of claim 1, wherein the biological specimen is selected from blood, tissue, serum, stool, urine, sputum, cerebrospinal fluid and supernatant from cell lysate.
3. The method of claim 1 wherein the neutrophilin is VEGF₁₆₅R/NP-1 or NP-2 or a homolog having at least about 90% homology to either.
4. A method for prognosing cancer in an individual having cancer comprising:
 - a. obtaining a tumor sample from said individual;
 - b. measuring VEGF₁₆₅R/NP-1 or NP-2 receptor amounts to obtain a receptor level in said sample;
 - c. correlating said receptor level with a baseline level; and
 - d. correlating levels of receptor greater than the baseline with an indication of unfavorable prognosis and levels of receptor at the baseline or less with a favorable prognosis.
5. The method of claim 1 or 4, wherein the cancer is prostate or breast.
6. A method for determining the metastatic potential of a tumor comprising measuring the level of VEGF₁₆₅R/NP-1 or NP-2 expression in said tumor, wherein levels of VEGF₁₆₅R/NP-1 or NP-2 in said tumor greater than a base line level indicates an increased metastatic potential.

7. The method of claim 3, 4 or 6 wherein the level of mRNA expressing VEGF₁₆₅R/NP-1 or NP-2 is measured.

8. The method of claim 7, wherein the mRNA is detected by use of an RNA dependent polymerase chain reaction.

9. The method of claim 3,4 or 5 wherein the level of the VEGF₁₆₅R/NP-1 or NP-2 is measured.

10. The method of claim 7, wherein the mRNA is detected by Northern blot analysis by hybridizing mRNA from said biological specimen to a VEGF₁₆₅ R/NP-1 or NP-2 nucleotide probe.

11. A method for measuring VEGF₁₆₅ R/NP-1 or NP-2 levels in a non-neuronal tissue which comprises the steps of:

a. contacting a sample or preparation thereof with an antibody or antibody fragment which selectively binds VEGF₁₆₅ R/NP-1 or NP-2, and

b. detecting whether said antibody or said antibody fragment is bound by said sample and thereby measuring the levels of VEGF₁₆₅ R/NP-1 or NP-2 present.

12. The method according to claim 11 wherein said antibody, or said antibody fragment, is detectably labeled.

Figure 1

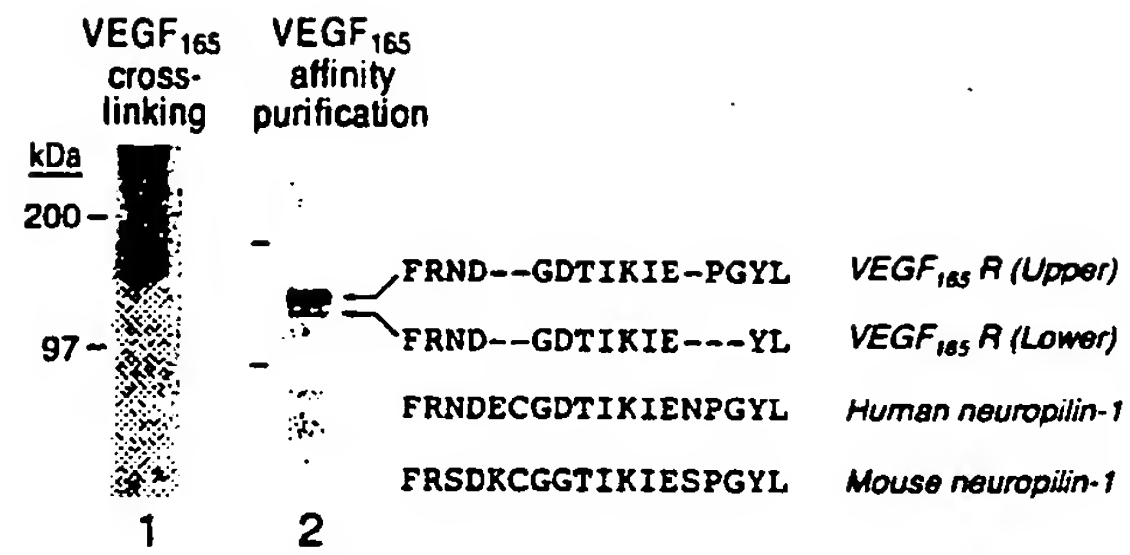
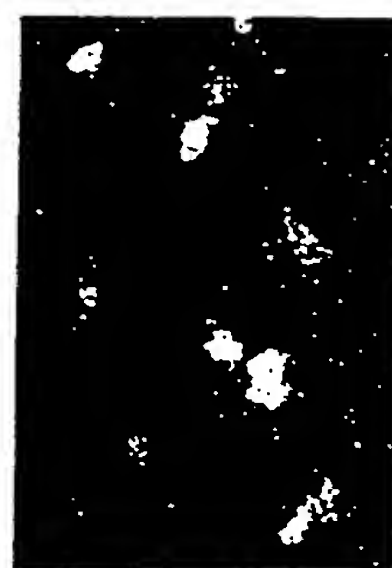


Figure 2A



Figure 2B



3/20

Figure 3

↓

1	MERGLPLLCAVLALVLAPAGAF	RNDKCGDTIKIESPGYLTS	PGYPHSYHPSEKCEWLIQAPDPYQIRIMIN	70			
71	FNPFDLEDROCKYDYVEVFDGENENGHFRGKFCGKIAPPPVVSSGPFLFIK	FVSDYETHGAGFSIRYEI		140			
141	FKRGPECSQNYTTTPSGVIKSPGFPEKYPNSLECTYIVFAPKMSEIILEFES	FDLEPDSNPPGGMFCRYDR		210			
211	LEIWDGFDPDVGPHIGRYCGQKTPGRIRSSSGILSMVFYTDSIAIAKEGFS	ANYSVLQSSVSEDFKCMEALG		280			
281	MESGEIHSDQITASSQYSTNWSAERSRLNYPENGWTPGEDSYREWIQVDL	GLLRFVTAVGTGGAISKETK		350			
351	KKYYVKTYKIDVSSNGEDWITIKEGNKPVLFQGNTPD	VVVAVFPKPLITRFVRIK	PATWETGISMRF	420			
421	VYGCKITDYPCSGMLGMVSGLISDSQITSSNQDRNWPENIRLVTSRSG	WALPPAPHSYIN	EWLQIDLG	490			
491	EEKIVRGIIIQGGKHRENKVFMRKFKIGYSNNGSDWKIMDDSKRKAKS	FEGNNNYDTPELRTFP	PALSTR	560			
561	FIRIYPERATHGGLGLRMELLGCEVEAPTAGPTTPGNL	VDECDDQANCHSGT	GDDFQLTGGTTVL	630			
631	KPTVIDSTIQSEFPTYGFNCEFGWGS	HKTFCHEHDNHVQLKWSVLT	SKTGPIQDHTG	DGNFIYSQADEN	700		
701	QKGKVARLVSPVVYSQNSAHCMTFWYHMSGSHVGT	LRVKLRYQKPEEYD	QLVWMAIGHQGDH	WKEGRVLL	770		
771	HKSLKLYQVIFEIGEIGKGNLGGIAVDDISINNHISQEDCAKPA	LDKKNPEIKIDETG	STPGYE	GEGE	840		
841	KNISRKPGNVLKTLDPILIT	IAMSALGVLLGAVCGV	LYCAC	WHNGMSE	RNLSALENYNFELVDG	VKLK	910
911	KDKLNTQSTYSEA	923					

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Figure 4 -

Comparative Deduced Amino Acid Sequences of Human VEGF₁₆₅R/NP and VEGF₁₆₅R/NP-1

VEGF ₁₆₅ R/NP-2	1	MDMF-PLTW-VFLALYFSRHQVRGQPPPCGG-RLNSK--DA-----GY	50
VEGF ₁₆₅ R/NP-1		MERGLPLLCAY-LAL-----VLA-PA---GAFR-NDKCGDTIKIESPGY	
NP-2	51	ITSPGYPDY-PSHQNCZW-IVYAPERNQKIVLNFNPHFEIEKHDCRYDF	100
NP-1		LTSPGYPHSYHPSEK-CEWLIQ-APDPYQRIMINFNPHFDLEDRCCKYDY	
NP-2	101	IZIRDGDSSESADLLGKHCGNIAPPTIISSGSMLYIKFTSDYARQGAGFSL	150
NP-1		VEVFDGENENGHFRGKFCGKIAPPPVYSSGPFLFIKFVSDYETHGAGFSI	
NP-2	151	RYZIFXTGSEDCSKNFTSPNGTIESPGFPEKYPHN-LDCTFTIL-AKPKM	200
NP-1		RYZIFKRGPE-CSQNYTTPSGVIKSPGFPEKYP-NSLECTY-IVFA-PKM	
NP-2	201	-EIILOFLIFDLEHD--PLQVGEGD-CKYDWLDIWDGIPHVGPPLIGKYCG	250
NP-1		SEIILEFESFDLEPDSNPP--G-GMFCRYDRLEIWDGFDDVGPPIGRYCG	
NP-2	251	TKTPSELRSSTGILSLTFHTDMAVAKOGESARYYLVHQEPL-ENFQCNVP	300
NP-1		QKTPGRIRSSSGILSMVFYTDSAIAKEGFSANYI-VLQSSVSDFKCMEA	
NP-2	301	LGMESGRIANEQISASSTYSJGRWTPQQRSLHGDDNGWTPNLDNKEYLQ	350
NP-1		LGMESGEIHSQITASSQYSTN-WSAERSRLNYPENGWTPGEDSYREWIQ	
NP-2	351	VDL---RFLTMTAIAATQGAISRETQNGYYVRSYKLEVSTNGEDWNVYRH	400
NP-1		VDLGLLRFTV---AVGTQGAISKETAKKYYVKTXYKIDVSSNGEDWITKE	
NP-2	401	GKNHK-V-FQAN-HDATEVVLN---KLHAPDLTRFVRIRPQTHSGIALR	450
NP-1		G-N-KPVLEQGNTHP-TDVVVAVFPEK---PLITRFVRIKPAWETGISM	
NP-2	451	LELFGCRVTDAPCSNMLGMLSGLIADSQISASSTQEYL-WSPSAARLVSS	500
NP-1		FEVYGCKITDYPCSGMLGMVSGLISDSQIT-SSNQGDNRWNMPENIRLVT	
NP-2	501	RSGWF-PRIPQAQPG---EWLQVDLGTPKTVKGVIIQGARGGDSITAVE	550
NP-1		RSGWALP--P-A-PHSYINELQIDLGEEXIVRGIIIQG--GKREKRV-	
NP-2	551	ARAFVRKFKVSYSLNGKDWETIDP--RTQQPRLFEGNMHYDTPDIRRED	600
NP-1		---FMRKFKIGYSNNGSDWXMIMDDSKRA--KSFEGNNHYDTPELRTF-	
NP-2	601	PIPAQYVRV---YPERWSPA--GI-GMRLEVLGCDWTDKPTVE--TLGP	650
NP-1		P--ALSTRFIRIYPER---ATHGGLGLRMELLGCE-----VEAPTAGP	
NP-2	651	TVKSEETTPPYPTTEATECGE---NC-SFE-DDKDLQ-----L----P-	700
NP-1		T-----T--PNGNLVD--ECDDQANCHSGTGDDFQLTGTTVLATEKPT	
NP-2	701	---S-----GFNCIFD-----FLEPCGWMYD-HA--KW--LRTT	750
NP-1		VIDSTIQSEFPTYGFNCEFGWGSHTF---CHWEHDNHVQLKNSVL-T-	
NP-2	751	WASSSSPN-DRTEPDNRFLRLQSDS-QREGQYARLISPPVHLPRSPVCH	800
NP-1		--SKTGPIQDHTG-DG-NFIYSQADENQK-GKVARLVSPVVYSQNSAHCM	
NP-2	801	EFQYQATG---G--RGVAL--QVVREASQESKLLWV-IREDDGGENKHKGR	850
NP-1		TFWYHMSGSHVGTLR-VKLRYQKPEEYDQ---LVWMAIGH-QGDHMKGR	
NP-2	851	IILP-SYDMEYQ-IVFEGVIGKGRSGEIAIDDIRI---STOVPLENCME	900
NP-1		VLLHKSLLK-YQVI-FEGEIGKGNLGGIAYDDISINNHISQ---EDCAR	
NP-2	901	PISAFAGENFKVDIPEIHERE-G----YEDEIDDEYVDWNSSSSATSGS	950
NP-1		P--ADLDK--KN--PEIKIDETGSTPGYEGEG--EG--DK-NISRKP-GH	
NP-2	951	GAPSTDKEKSWLYTLDPILITIIAMSSLGVLGATCAGLLLYCTCSYSGL	1000
NP-1		VL----K-----TLDPILITIIAMSA LGVLLGAVC-GVVLYCACWNGM	
NP-2	1001	SSR--SCITLENYNFELYDG--LKHKVRMNHQKCCSEA	1038
NP-1		SERNLSA--LENYNFELVDGVKLG-KDRINTQSTYSEA	

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Figure 5

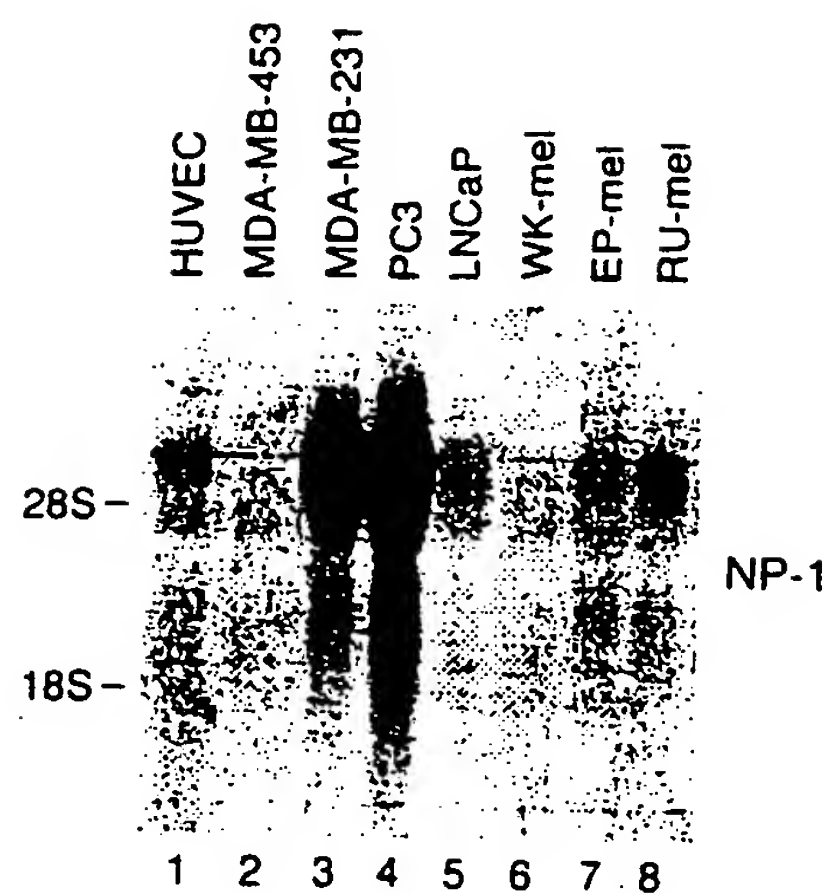


Figure 6

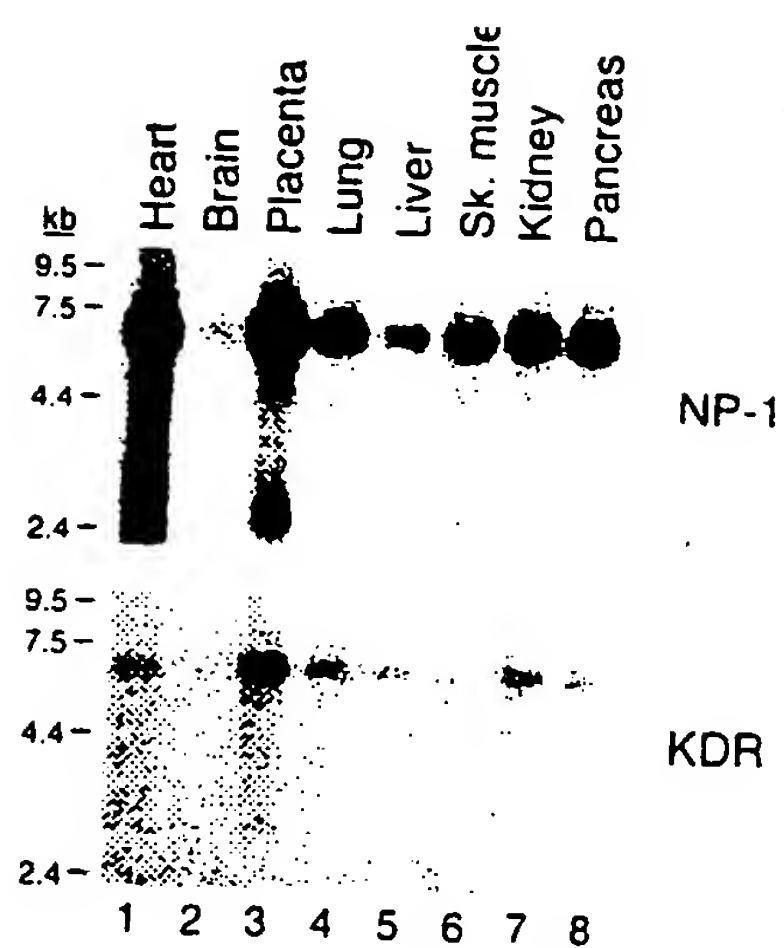


Figure 7A

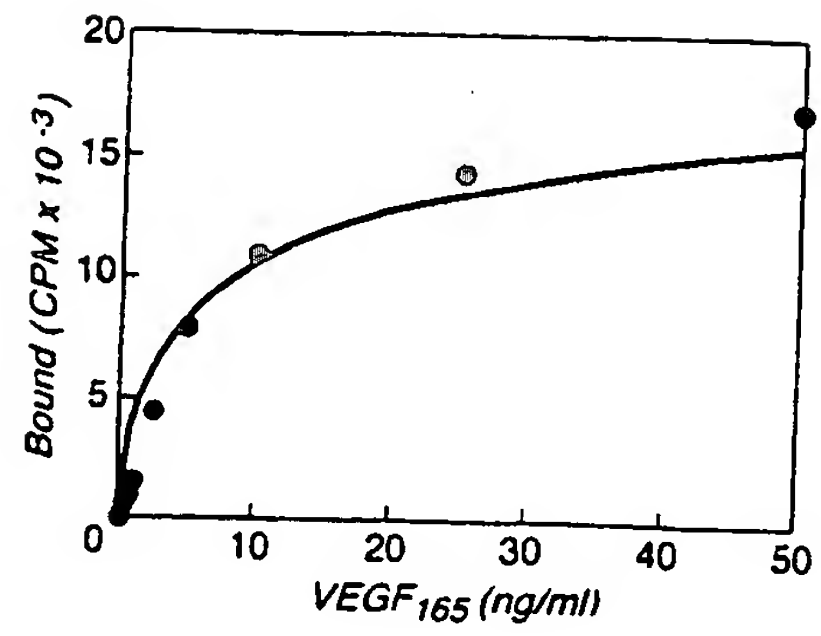


Figure 7B

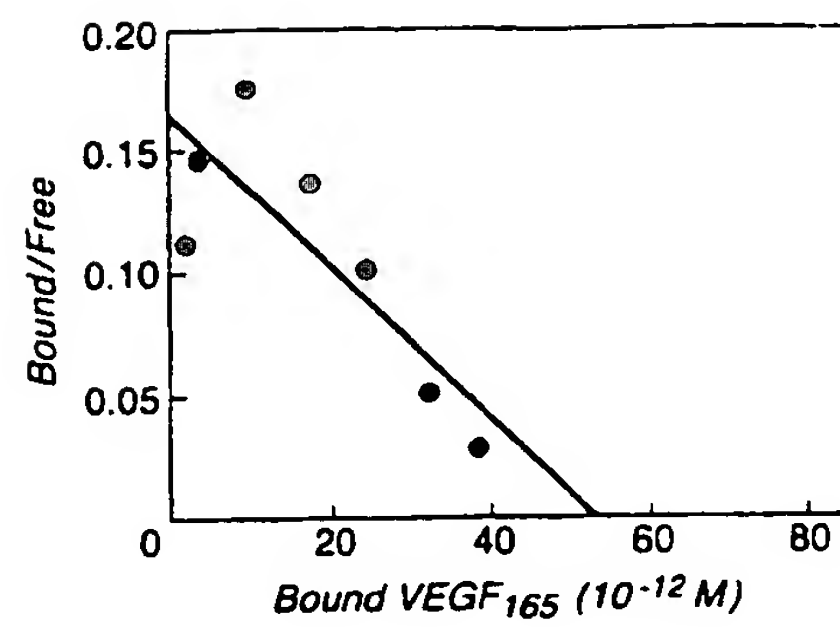


Figure 8

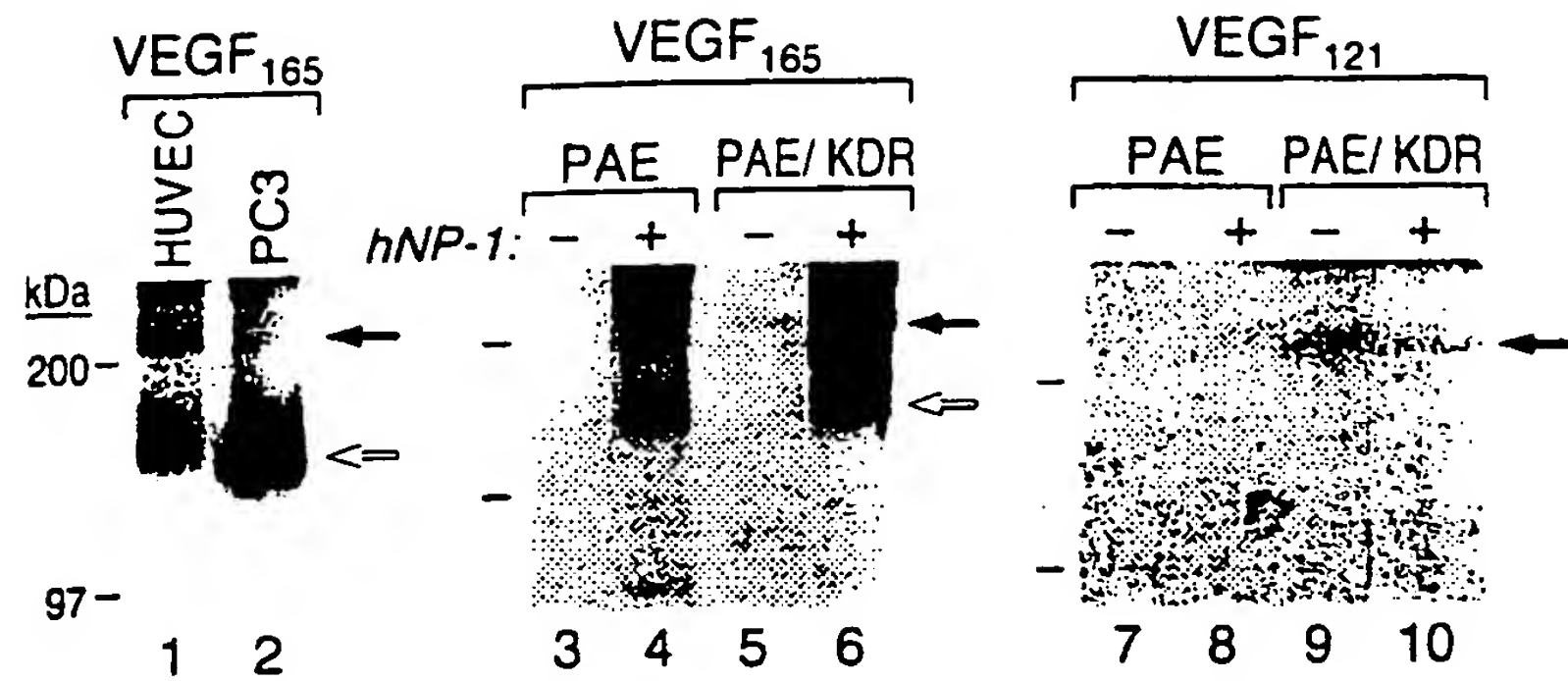


Figure 9

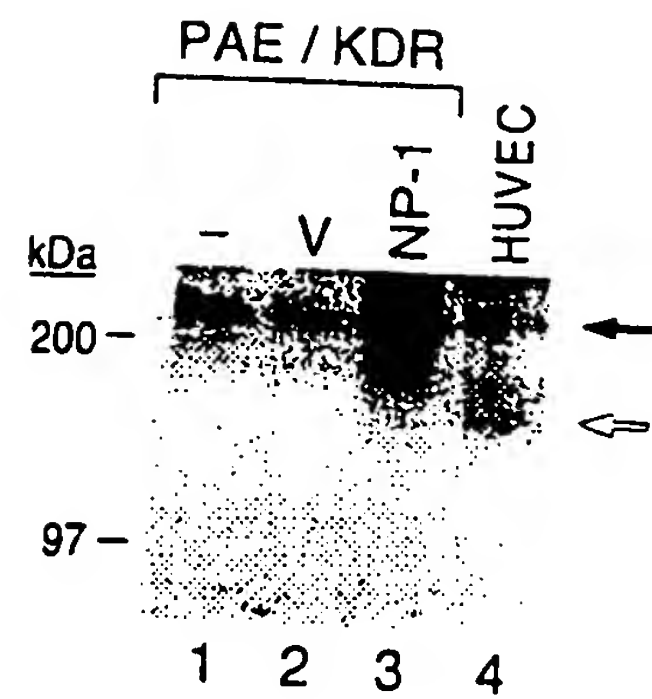


Figure 10

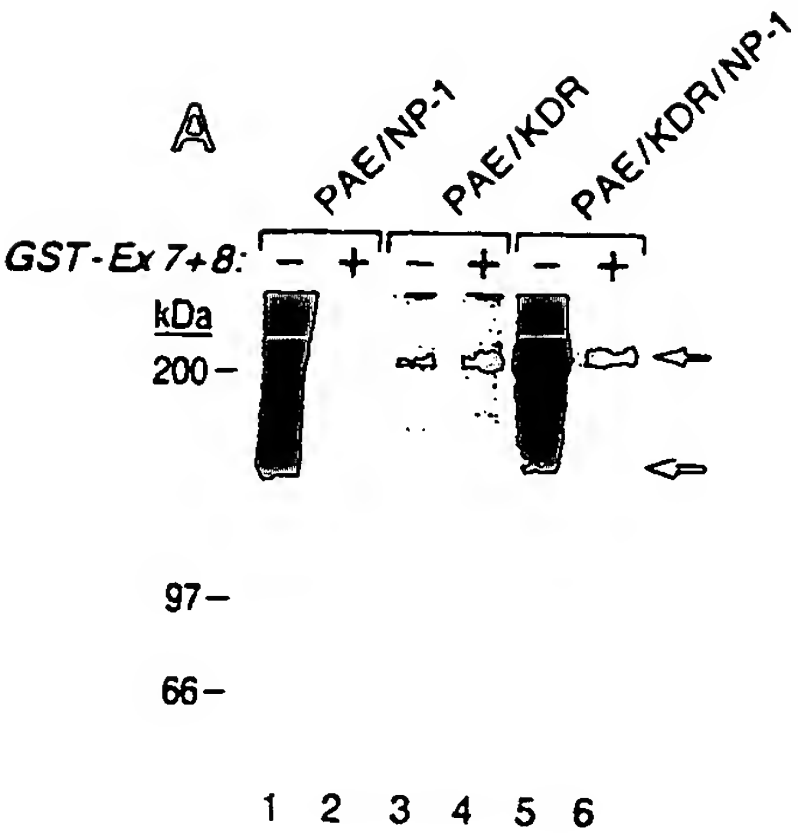


Figure 11A

KDR alone

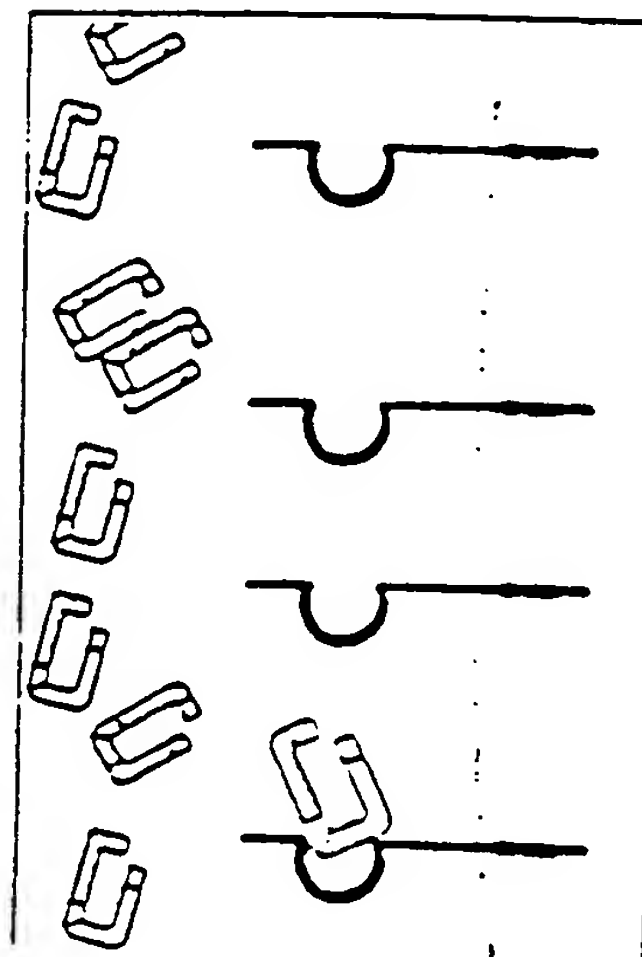
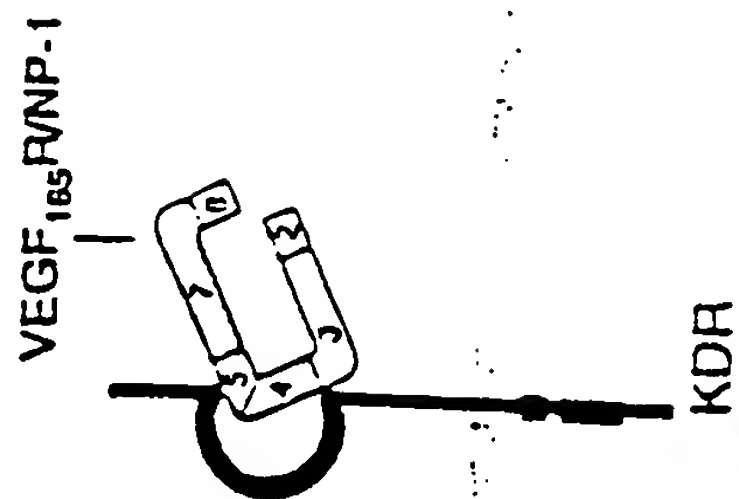


Figure 11B

KDR + VEGF₁₆₅R/NP-1

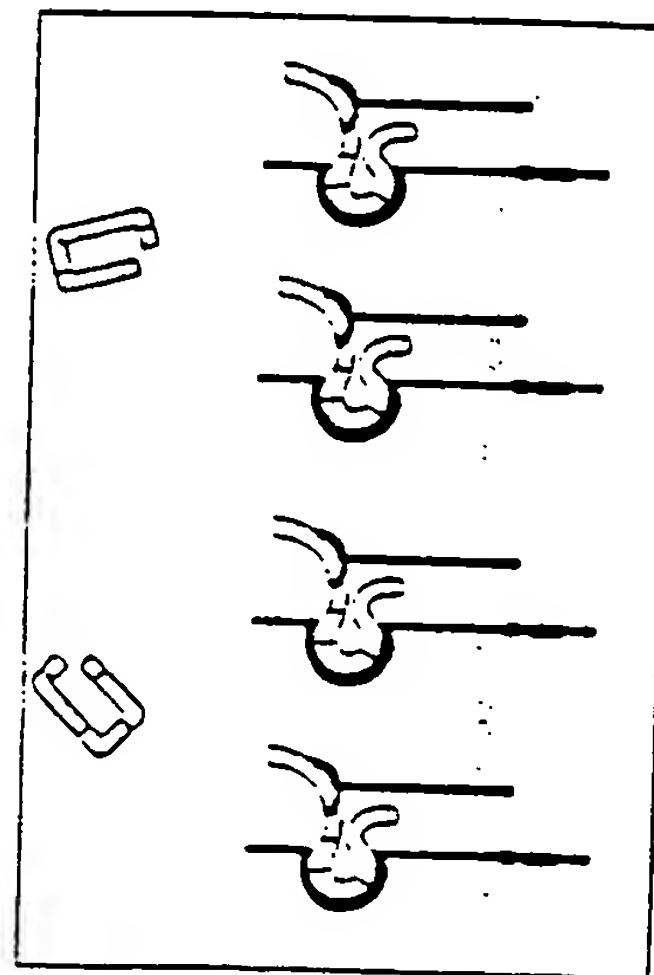
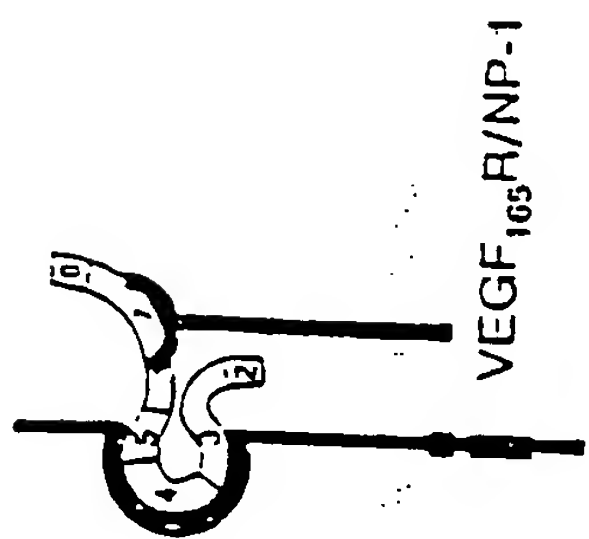


Figure 11C
KDR + VEGF₁₆₅R/NP-1
+ Exon 7

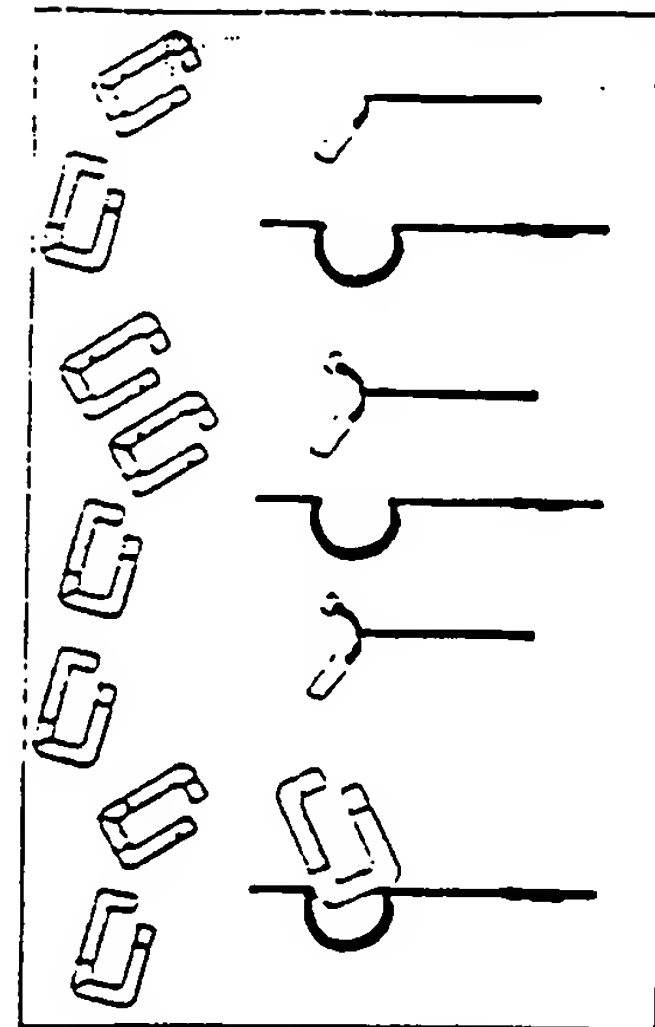
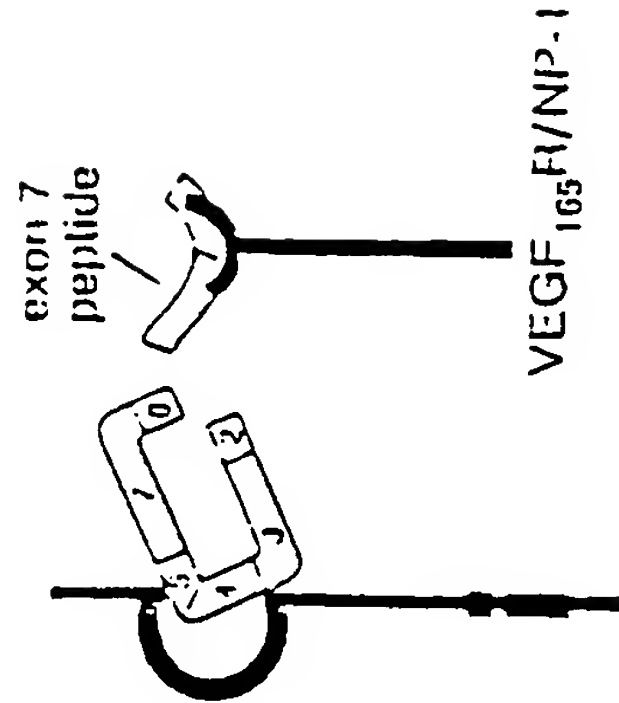


Figure 12

Human Neuropilin-2 amino acid sequence:

MDMFPLTWVFLALYFSRHQVRGQPDFPCGGRLNSKDAGYITSPGY PQDYPSHQN
CEWVYAPEPNCKIVLNFNPHFEIEKH DCKYDFEIRDGDSE S ADLLGKHCGNIAPP
TISSGSMLYIKFTSDYARQGAGFSLRYEIFKTGSEDCSKNFTSPNGTIESFGFPEK
YPHNLDCTFTILAKPKMEIILQFLIFDLEH DPLQVGEGDCKYDWLDIWDGIPHVGPL
IGKYCGTKTPSELRSSTGILSLTFHTDMAVAKDGFSARYYL VHQEPLENFQCNVP
LGMESGRIANEQISASSTYS DGRWTFQCSRLHGDDNGWTFNLDSNKEYLQVDLR
FLTMLTAIATQGAISRETQNGYYVKS YKLEVSTNGEDWMVYRHGKNHKVFQANN
DATEVVLNKLHAPLLTRFVRIRPQTWHSGIALRL ELFGCRVTDAPCSNMLGMILS
GLIADSQISASSTQEYLWSPSAARLVSSRSGWFPRIPOA.QPGEEWLQVDLGTPK
TVKGVIIQGARGGDSITAVEARAFVRKFKVSYSLNGKDWEYIQDPRTQQPKLFEG
NMHYDTPDIRRFDPIPAQYVRVYPERWSPAGIGMRLEV LGCDWTD SKPTVETLG
PTVKSEETTTPTYPTEEEATECGENC SFEDDKDLQLFSGFNCNFD FLEEPCGWMYD
HAKWLRTTWASSSSPNDRTFPDDRNFLRLQSDSQREGQYARLISP PVHLPRSPV
CMEFQYQATGGRGVALQVVREASQESKLLWVIRE DQGGEWKHGRILLPSYDMIEYQ
IVFEGVIGKGRSGEIAIDDIRISTDVPLENCMEPISAFAGENFKVDIPEIHEREGYED
EIDDEYEVDWSNSSSATSGSGAPSTDKEKSWLYTLDPILITIIAMSS LGVLLGAT
CAGLLLYCTCSYSGLSSRSCTTLENYNFELYDGLKHKVKMINHQKCCSEA*

Figure 13

gaattcggca	cgaggggaaa	ataaaagaga	gaaaaacaca	aagattttaa	caagaaacct	60
acgaacccag	ctctggaaag	agccaccttc	tccaaaatgg	atatgtttcc	tctcacctgg	120
gttttcttag	ccctctactt	ttcaagacac	caagtgaag	gccaaccaga	cccaccgtgc	180
ggaggtcgtt	tgaattccaa	agatgctggc	tatatcacct	ctcccgggta	ccccaggac	240
tacccctccc	accagaactg	cgagtggatt	gtttacgccc	ccgaacccaa	ccagaagatt	300
gtcctcaact	tcaaccctca	ctttgaaatc	gagaagcacg	actgcaagta	tgactttatc	360
gagattcggg	atggggacag	tgaatccgca	gacctcctgg	gcaaactactg	tggaacatc	420
gccccgccc	ccatcatctc	ctcgggctcc	atgctctaca	tcaagtccac	ctccgactac	480
gcccggcagg	gggcaggctt	ctctctgcgc	tacgagatct	tcaagacagg	ctctgaagat	540
tgctcaaaaa	acttcacaag	ccccaacggg	accatcgaat	ctcctgggtt	tcctgagaag	600
tatccacaca	acttggaactg	cacctttacc	atcctggcca	aaccaagat	ggagatcatc	660
ctgcagttcc	tgatctttga	cctggagcat	gaccttttgc	aggtgggaga	gggggactgc	720
aagtacgatt	ggctggacat	ctgggatggc	attccacatg	ttggccccct	gattggcaag	780
tactgtggga	ccaaaacacc	ctctgaactt	cgttcatcga	cggggatcct	ctccctgacc	840
tttcacacgg	acatggcggg	ggccaaggat	ggcttctctg	cgcgttacta	cctggtccac	900
caagagccac	tagagaactt	tcagtgcgat	gttctctctg	gcatggagtc	tgcccgatt	960
gctaataaac	agatcagtgc	ctcatctacc	tactctgatg	ggaggtggac	ccctcaacaa	1020
agccggctcc	atggtgatga	caatggctgg	acccccaact	tggaattccaa	caaggagtat	1080
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tggggaagat	tacatttttt	tttccttttg	aaactgaatg	ccataatctc	gatcaaaccg	3000
atccagaata	ccgaaggat	ggacaggaca	gaaaagcgag	tcgcaggagg	aaggagagatg	3060
cagccgcaca	ggggatgatt	accctcctag	gaccgcgggtg	gctaagtcac	tgaggaacg	3120
gggctgtgtt	ctctgctggg	acaaaacagg	agctcatctc	tttgggggtca	cagttctatt	3180
ttgtttgtga	gtttgtatta	ttattattat	tattattatt	atattttatt	tctttgtctt	3240
gtgagcaact	caaagaggca	gaagaggaga	atgacttttc	cagaatagaa	gtggagcagt	3300
gatcattatt	ctccgctttc	tctttcta	caacacttga	aaagcaaagt	gtcttttcag	3360
cctttccatc	tttacaata	aaactcaaaa	aagctgtcca	gctt		3404

Figure 14A

5' ATG GAG AGG GGG CTG CCG CTC CTC TGC GCC GTG CTC GCC CTC GTC CTC GCC CCG
 M E R G L P L L C A V I A L V L A P

 GCC GGC GCT TTT CGC AAC GAT AAA TGT GGC GAT ACT ATA AAA ATT GAA AGC CCC
 A G A ^LF ^RN D K C G D T I K I E S P

 GGG TAC CTG ACA TCT CCG GGT TAT CCT CAT TCT TAT CAC CCA AGT GAA AAA TGC
 G Y L T S P G Y P H S Y H P S E K C

 GAA TGG CTG ATT CAG GCT CCG GAC CCA TAC CAG AGA ATT ATG ATC AAC TTC AAC
 E W L I Q A P D P Y Q R I M I N F N

 CCT CAC TTC GAT TTG GAG GAC AGA GAC TGC AAG TAT GAC TAC GTG GAA GTG TTC
 P H F D L E D R D C K Y D Y V E V F

 GAT GGA GAA AAT GAA AAT GGA CAT TTT AGG GGA AAG TTC TGT GGA AAG ATA GCC
 D G E N E N G H F R G K F C G K I A

 CCT CCG CCT GTT GTG TCT TCA GGG CCA TTT CTT TTT ATC AAA TTT GTC TCT GAC
 P P P V V S S G P F L F I K F V S D

 TAC GAA ACA CAT GGT GCA GGA TTT TCC ATA CGT TAT GAA ATT TTC AAG AGA GGT
 Y E T H G A G F S I R Y E I F K R G

 CCT GAA TGT TCC CAG AAC TAC ACA ACA CCG AGT GGA GTG ATA AAG TCC CCC GGA
 P ^EC ^SQ N Y T T P S G V I K S P G

 TTC CCT GAA AAA TAT CCC AAC AGC CTT GAA TGC ACT TAT ATT GTC TTT GCG CCA
 F P E K Y P N S L E C T Y I V F A P

 AAG ATG TCA GAG ATT ATC CTG GAA TTT GAA AGC TTT GAC CTG GAG CCT GAC TCA
 K M S E I I L E F E S F D L E P D S

 AAT CCT CCA GGG GGG ATG TTC TGT CGC TAC GAC CGG CTA GAA ATC TGG GAT GGA
 N P P G G M F C R Y D R L F I W D G

 TTC CCT GAT GTT GGC CCT CAC ATT GGG CGT TAC TGT GGA CAG AAA ACA CCA GGT
 F P D V G P H I G R Y C G Q K T P G

 CGA ATC CGA TCC TCA TCG GGC ATT CTC TCC ATG GTT TTT TAC ACC GAC AGC GCG
 R I R S S S G I L S M V F Y T D S A

- June 14^B -

1003	1012	1021	1030	1039	1048
ATA GCA AAA GAA GGT TTC TCA GCA AAC TAC AGT GTC TTG CAG ACC AGT GTC TCA					
I A K E G F S A N Y S V L Q S S V S					
1057	1066	1075	1084	1093	1102
GAA GAT TTC AAA TGT ATG GAA GCT CTG GGC ATG GAA TCA GGA GAA ATT CAT TCT					
E D F K C H E A L G M E S G E I H S					
1111	1120	1129	1138	1147	1156
GAC CAG ATC ACA GCT TCT TCC CAG TAT AGC ACC AAC TGG TCT GCA GAG CGC TCC					
D Q I T A S S Q Y S T N W S A E R S					
1165	1174	1183	1192	1201	1210
CGC CTG AAC TAC CCT GAG AAT GGG TGG ACT CCC GGA GAG GAT TCC TAC CGA GAG					
R L N Y P E N G W T P G E D S Y R E					
1219	1228	1237	1246	1255	1264
TGG ATA CAG GTA GAC TTG GGC CTT CTG CGC TTT GTC ACG GCT GTC GGG ACA CAG					
W I Q V D L G L L R F V T A V G T Q					
1273	1282	1291	1300	1309	1318
GGC GCC ATT TCA AAA GAA ACC AAG AAG AAA TAT TAT GTC AAG ACT TAC AAG ATC					
G A I S K E T K K K Y Y V K T Y K I					
1327	1336	1345	1354	1363	1372
GAC GTT AGC TCC AAC GGG GAA GAC TGG ATC ACC ATA AAA GAA GGA AAC AAA CCT					
D V S S N G E D W I T I K E G N K P					
1381	1390	1399	1408	1417	1426
GTT CTC TTT CAG GGA AAC ACC AAC CCC ACA GAT GTT GTG GTT GCA GTA TTC CCC					
V L F Q G N T N P T D V V V A V F P					
1435	1444	1453	1462	1471	1480
AAA CCA CTG ATA ACT CGA TTT GTC CGA ATC AAG CCT GCA ACT TGG GAA ACT GGC					
K P L I T R F V R I K P A T W E T G					
1489	1498	1507	1516	1525	1534
ATA TCT ATG AGA TTT GAA GTA TAC GGT TGC AAG ATA ACA GAT TAT CCT TGC TCT					
I S M R F E V Y G C K I T D Y P C S					
1543	1552	1561	1570	1579	1588
GGA ATG TTG GGT ATG GTG TCT GGA CTT ATT TCT GAC TCC CAG ATC ACA TCA TCC					
G M L G M V S G L I S D S Q I T S S					
1597	1606	1615	1624	1633	1642
AAC CAA GGG GAC AGA AAC TGG ATG CCT GAA AAC ATC CGC CTG GTA ACC AGT CGC					
N Q G D R N W M P E N I R L V T S R					
1651	1660	1669	1678	1687	1696
TCT GGC TGG GCA CTT CCA CCC GCA CCT CAT TCC TAC ATC AAT GAG TGG CTC CAA					
S G W A L P P A P H S Y I N E W L Q					
1705	1714	1723	1732	1741	1750
ATA GAC CTG GGG GAG GAG AAG ATC GTG AGG GGC ATC ATC ATT CAG GGT GCG AAG					
I D L G E E K J V R G I I I Q G G K					
1759	1768	1777	1786	1795	1804
CAC CGA GAG AAC AAG GTG TTC ATG AGG AAG TTC AAG ATC GGG TAC AGC AAC AAC					
H R E N K V F M R K F K I G Y S N N					

figure 14b

1813	1822	1831	1840	1849	1858
GGC TCG GAC TGG	AAG ATG ATC ATG GAT	GAC AGC AAA CGC	AAG GCG AAG TCT	TTT	
G S D W	K M I M D D	S K R K A K	S F		
1867	1876	1885	1894	1903	1912
GAG GGC AAC AAC	AAC TAT GAT ACA CCT	GAG CTG CGG ACT	TTT CCA GCT	CTC TCC	
E G N N N Y D T P	E L R T F P A L	S			
1921	1930	1939	1948	1957	1966
ACG CGA TTC ATC	AGG ATC TAC CCC	GAG AGA GCC ACT	CAT GGC GGA	CTG GGG	CTC
T R F I R I Y P F	R A T H G G L G L				
1975	1984	1993	2002	2011	2020
AGA ATG GAG CTG	CTG GGC TGT GAA GTG	GAA GCC CCT	ACA GCT GGA	CCG ACC	ACT
R M E L L G C E V	A P T A G P T T				
2029	2038	2047	2056	2065	2074
CCC AAC GGG AAC	TTG GTG GAT GAA TGT	GAT GAC GAC CAG	GCC AAC TGC	CAC AGT	
P N G N L V D E C D D D	Q A N C H S				
2083	2092	2101	2110	2119	2128
GGA ACA GGT GAT	GAC TTC CAG CTC	ACA GGT GGC ACC	ACT GTG CTG	GCC ACA	GAA
G T G D D F Q L T G G T T	V L A T E				
2137	2146	2155	2164	2173	2182
AAG CCC ACG GTC	ATA GAC AGC ACC	ATA CAA TCA	GAG TTT CCA	ACA TAT	GGT TTT
K P T V I D S T I Q S E F P T Y	L G C				
2191	2200	2209	2218	2227	2236
AAC TGT GAA TTT	GGC TGG GGC TCT	CAC AAG ACC TTC	TGC CAC TGG	GAA CAT	GAC
N C E F G W G S H K T F C H W E H D					
2245	2254	2263	2272	2281	2290
AAT CAC GTG CAG	CTC AAG TGG AGT GTG	TTG ACC AGC AAG	ACG GGA CCC	ATT CAG	
N H V Q L K W S V L T S K T G P I O					
2299	2308	2317	2326	2335	2344
GAT CAC ACA GGA	GAT GGC AAC TTC	ATC TAT TCC CAA	GCT GAC GAA	AAT CAG	AAG
D H T G D G N F I Y S Q A D E N Q K					
2353	2362	2371	2380	2389	2398
GGC AAA GTG GCT	CGC CTG GTG AGC	CCT GTG GTT	TAT TCC CAG	AAC TCT	GCC CAC
G K V A R L V S P V V Y S Q N S A H					
2407	2416	2425	2434	2443	2452
TGC ATG ACC TTC	TGG TAT CAC ATG	TCT GGG TCC	CAC GTC GGC	ACA CTC	AGG GTC
C M T F W Y H M S G S H V G T L R V					
2461	2470	2479	2488	2497	2506
AAA CTG LGC TAC	CAG AAG CCA GAG	GAG TAC GAT	CAG CTG GTC	TGG ATG	GCC ATT
K L R Y Q K P E E Y D Q L V W M A I					
2515	2524	2533	2542	2551	2560
GGA CAC CAA GGT	GAC CAC TGG AAG	GAA GGG CGT	GTC TTG CTC	CAC AAG	TCT CTG
G H Q G D H W K E G R V L L H K S L					
2569	2578	2587	2596	2605	2614
AAA CTT TAT CAG	GTG ATT TTC GAG	GGC GAA ATC	GGG AAA GGA	AAC CTT	GGT GGG
K L Y Q V T F E G E I G K G N L G G					

Figure 14D

2623	2632	2641	2650	2659	2668
ATT GCT GTG GAT GAC ATT	AGT ATT AAC AAC CAC ATT	TCA CAA GAA GAT TGT GCA			
I A V D D I S I N N H I S Q E D C A					
2677	2686	2695	2704	2713	2722
AAA CCA GCA GAC CTG GAT	AAA AAG AAC CCA GAA ATT	AAA ATT GAT GAA ACA GGG			
K P A D L D K K N P E I K I D F T G					
2731	2740	2749	2758	2767	2776
AGC ACG CCA GGA TAC GAA	GGT GAA GGA GAA GGT GAC	AAG AAC ATC TCC AGG AAG			
S T P G Y E G E G E G D K N I S R K					
2785	2794	2803	2812	2821	2830
CCA GGC AAT GTG TTG AAG	ACC TTA GAT CCC ATC CTC	ATC ACC ATC ATA GCC ATG			
P G N V L K T L D P I T I I A M					
2839	2848	2857	2866	2875	2884
AGT GCC CTG GGG GTC CTC	CTG GGG GCT GTC TGT GGG	GTC GTG CTG TAC TGT GCC			
S A L G V L L G A V C G V V L Y C A					
2893	2902	2911	2920	2929	2938
TGT TGG CAT AAT GGG ATG	TCA GAA AGA AAC TTG TCT	GCC CTG GAG AAC TAT AAC			
C _{TM} W _{II} C _N G M S E R N L S A L E N Y N					
2947	2956	2965	2974	2983	2992
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3001	3010				
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Figure 15A

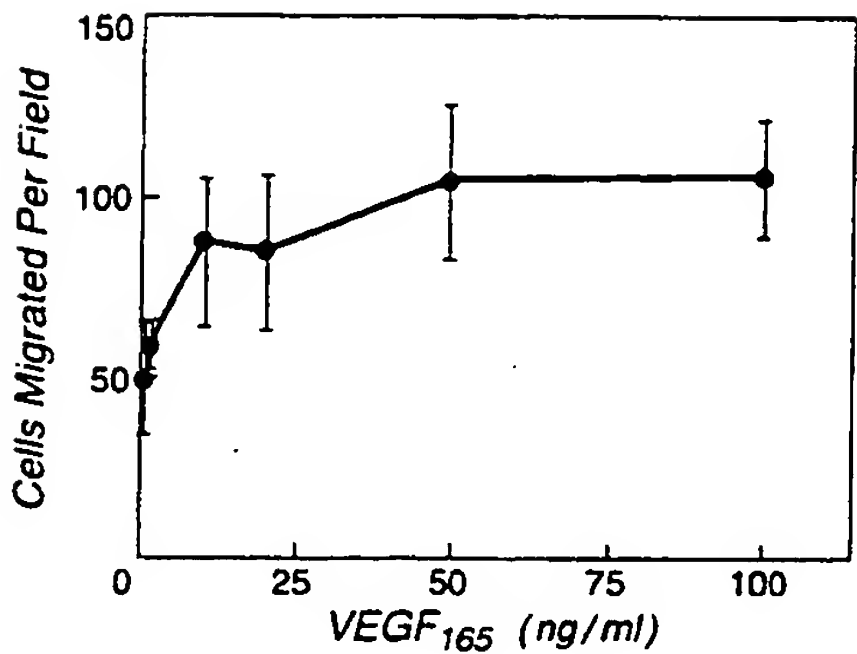


Figure 15B

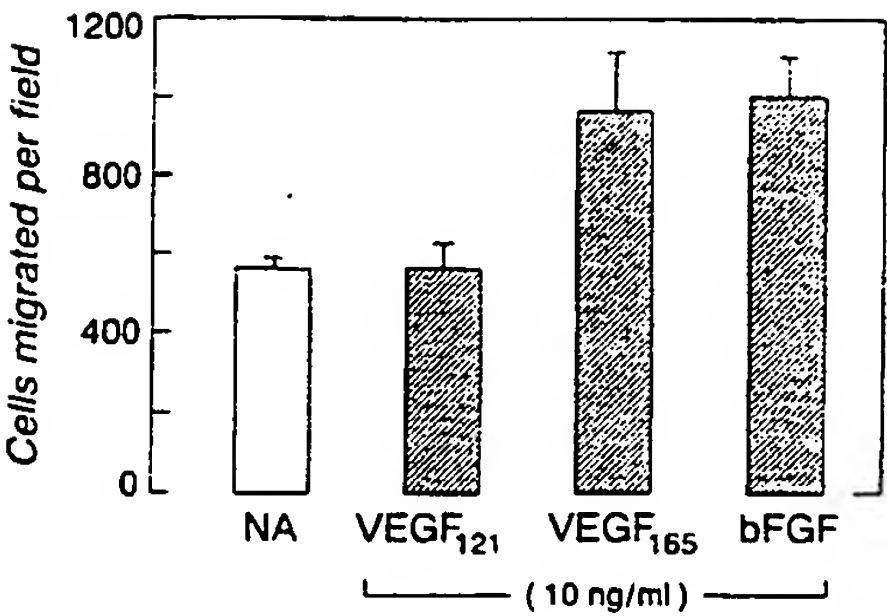


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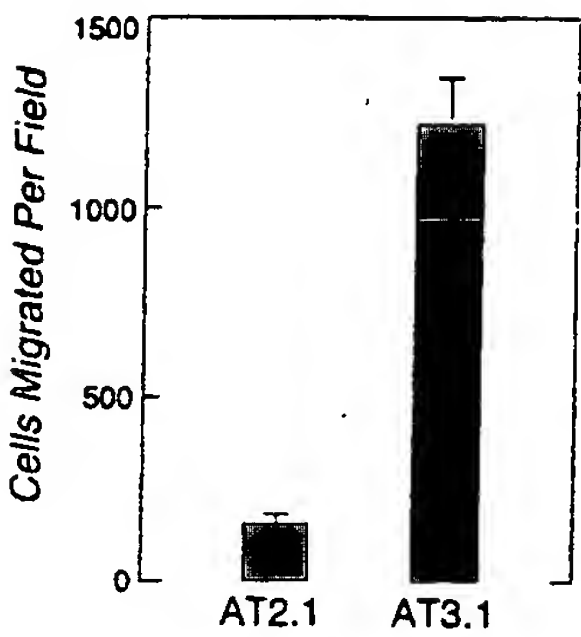


Figure 16B

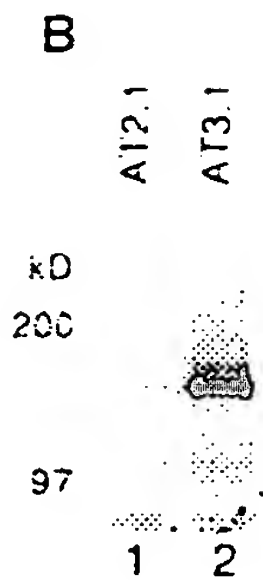


Figure 16C

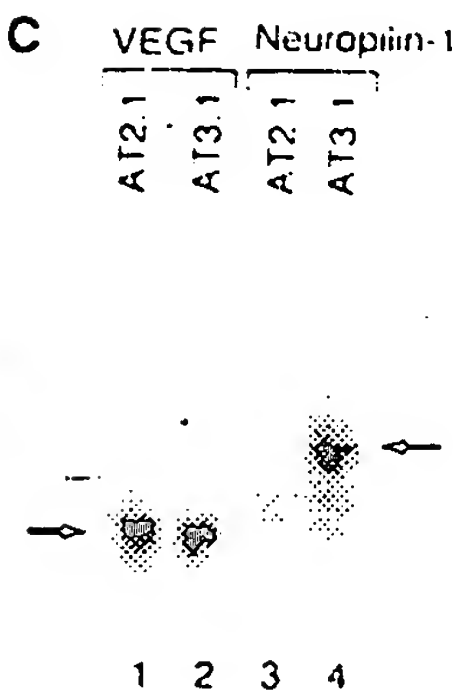


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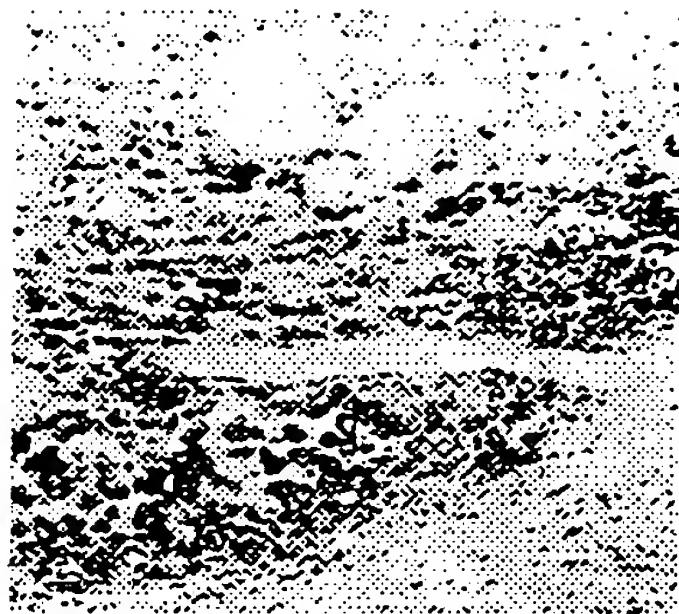


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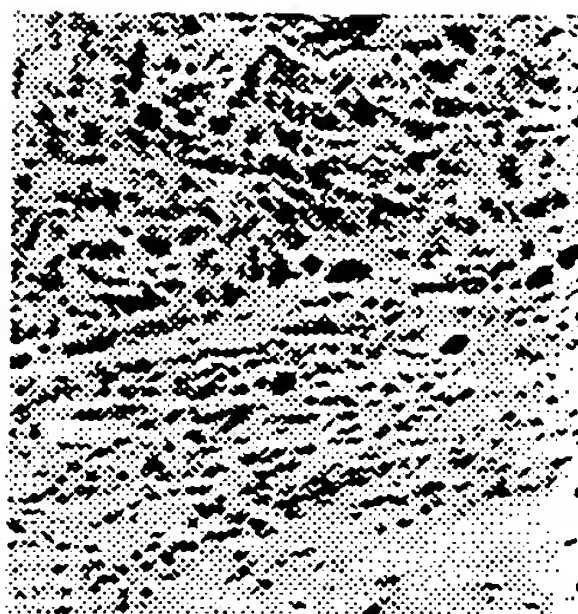


Figure 17C



Figure 18A

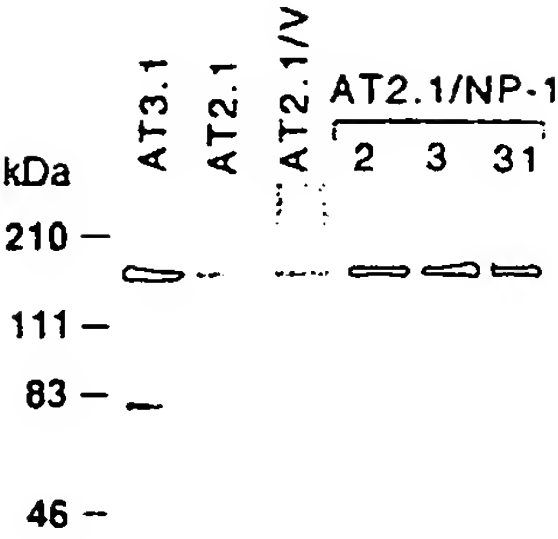
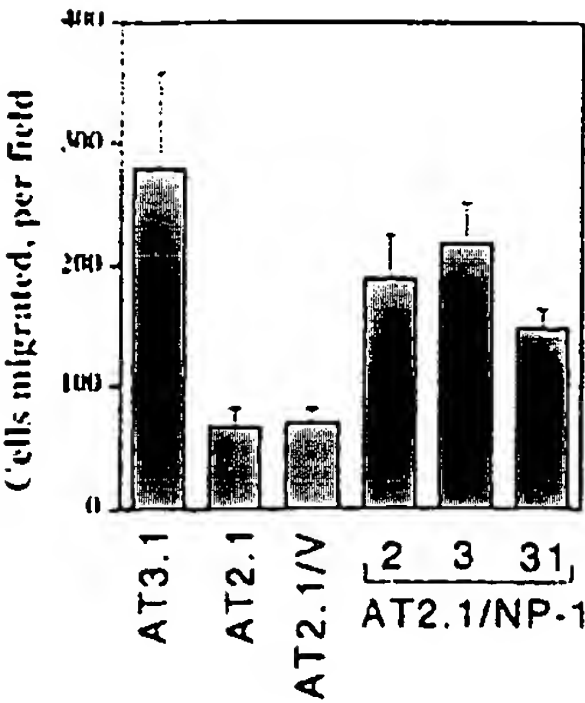
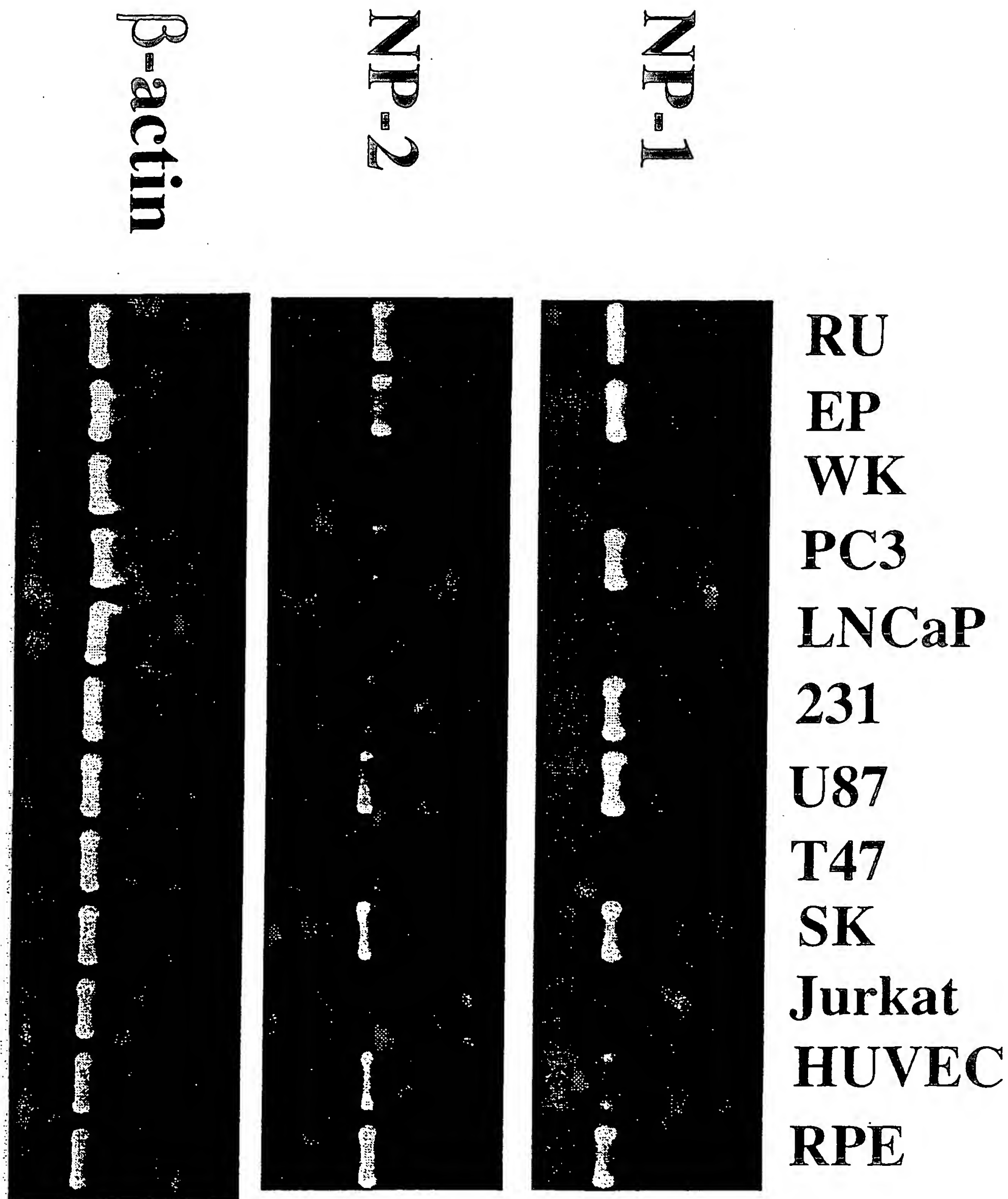


Figure 18B





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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US98/26127 (22) International Filing Date: 9 December 1998 (09.12.98) (30) Priority Data: 60/069,155 9 December 1997 (09.12.97) US 60/069,687 12 December 1997 (12.12.97) US (71) Applicant (for all designated States except US): CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 320 Longwood Avenue, Boston, MA 02115 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): KLAGSBRUN, Michael [US/US]; 22 Berwick Road, Newton, MA 02159 (US). SOKER, Shay [IL/US]; 92 Beals Street, Brookline, MA 02446 (US). MIAO, Hua-Quan [CN/US]; Apartment 8, 50 Babcock Street, Brookline, MA 02446 (US). TAKASHIMA, Seiji [JP/JP]; 1st dept. of Internal Medicine, Osaka University School of Medicine, 2-2, Yamadoaka Suita, Osaka 565-0871 (JP). (74) Agents: RESNICK, David, S. et al.; Peabody & Brown, 101 Federal Street, Boston, MA 02110-1832 (US).		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 22 July 1999 (22.07.99)
(54) Title: NEUROPILINS IN METHODS FOR DIAGNOSIS AND PROGNOSIS OF CANCER (57) Abstract The present invention relates to VEGF receptors (VEGFR) and neuropilins such as VEGF ₁₆₅ R/NP-1 and NP-2 that are associated with metastatic potential of a malignant cell and their use in the diagnosis and prognosis of cancer. Preferred ones are VEGF ₁₆₅ R/NP-1 and NP-2 but any neuropilin or VEGFR, where the constituents share at least about 85 % homology with either of the above VEGF ₁₆₅ R/NP-1 and NP-2 can be used. More preferably, such constituent shares at least 90 % homology. Still more preferably, each constituent shares at least 95 % homology.		

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/26127

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/574 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	S. SOKER ET AL.: "Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor." CELL, vol. 92, 20 March 1998, pages 735-745, XP002097906 see the whole document ---	1-12
Y	S. SOKER ET AL.: "Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 10, 8 March 1996, pages 5761-5767, XP002097905 cited in the application see the whole document ---	1-12
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

19 May 1999

Date of mailing of the international search report

01/06/1999

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Griffith, G

INTERNATIONAL SEARCH REPORT

Int. l. Application No
PCT/US 98/26127

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>T. OMURA ET AL.: "Identification of a 190-kDa vascular endothelial growth factor 165 cell surface binding protein on a human glioma cell line." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 37, 12 September 1997, pages 23317-23322, XP002097907 cited in the application see the whole document ---</p>	1-12
Y	<p>WO 94 11499 A (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFT E.V.) 26 May 1994 see page 39, line 5 - page 47, line 31 ---</p>	1-12
Y	<p>WO 97 08313 A (GENENTECH, INC.) 6 March 1997 see page 45, line 7 - line 28 ---</p>	1-12
A	<p>H. CHEN ET AL.: "Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins sema E and sema IV but not sema III." NEURON, vol. 19, September 1997, pages 547-559, XP002084494 cited in the application ---</p>	
A	<p>A. L. KOLODKIN ET AL.: "Neuropilin is a semaphorin III receptor." CELL, vol. 90, 22 August 1997, pages 753-762, XP002084496 cited in the application -----</p>	

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information on patent family members

International Application No

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		EP 0669978 A	06-09-1995
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